THE ROLE OF THE CHICKEN GONADOTROPIN-RELEASING HORMONE RECEPTOR C-TERMINAL TAIL IN EXPRESSION AND COUPLING

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LIST OF ABREVIATIONS:

AT_{1A}-R:	type 1a angiotensin receptor
α_1-AR :	type 1 adrenergic receptor
$\alpha_{2\alpha}$-AR:	type 2a adrenergic receptor
α_{1B}-AR:	type 1B adrenergic receptor
αT3–1 :	stable mouse gonadotrope cell line
β_2-AR :	type 2 β adrenergic receptor
Ca^{2+}:	calcium
cAMP:	adenosine 3',5' cyclic monophosphate
cDNA:	complementary DNA
cfGnRHR:	catfish GnRHR
cGMP:	guanosine 3',5' cyclic monophosphate
cGnRHR:	chicken GnRHR
COS cells:	stable monkey kidney cells
C-terminal:	carboxy terminal
DAG:	diacylglycerol
EC_{50}:	concentration of ligand that produces half the maximal response from the receptor
ECL:	extracellular loop
EP3β-R:	β-type prostaglandin E receptor
ER:	endoplasmic reticulum
FCS:	fetal calf serum
FSH:	follicle-stimulating hormone
FSHR:	follicle stimulating hormone receptor
GDP:	guanosine-5'-diphosphate
GfaGnRHR:	goldfish type a GnRHR
GfbGnRHR:	goldfish type b GnRHR
GGH3 cells:	GH3 cells stably transfected with rGnRHR
GH3 cells:	pituitary-derived lactotrope cell line
GnRH:	gonadotropin-releasing hormone
GnRHR:	gonadotropin releasing hormone receptor
GPCR:	G-protein coupled receptor
G-proteins:	GTP-binding protein

GRKs:	G-protein coupled receptor kinases
GTP:	guanosine-5'-trisphosphate
HEK293:	stable human embryonic kidney cells
hGnRHR:	human GnRHR
H_2-R:	type 2 histamine receptor
IC_{50}:	dose required to occupy half the maximal amount of receptors
ICL:	intracellular loop
IP_3:	inositol 1,4,5-trisphosphate
IPs:	inositol phosphates
JNK:	Jun N-terminal kinase
LH:	luteinizing hormone
LHR:	luteinizing hormone receptor
MAPK:	mitogen-activated protein kinase
mGnRHR:	mouse GnRHR
NK_2-R:	type 2 neurokinin receptor
N-terminal:	amino terminal
PCR:	polymerase chain reaction
PIP_2:	phosphatidylinositol 4,5-bisphosphate
PKC:	protein kinase C
PLC-β:	β phosphoinositide specific phospholipase C
PS:	penicillin and streptomycin
PTX:	pertussis toxin
~Q:	relative coupling efficiency
rGnRHR:	rat GnRHR
Rh-R:	rhodopsin receptor
TGN:	trans-golgi network
TM:	transmembrane
TRHR:	thyroid releasing hormone receptor
TSHR:	thyroid-stimulating hormone receptor
V1a-R:	type 1a vasopressin receptor
V2-R:	type 2-vasopressin receptor
wt:	wild type

1. ABSTRACT:

The role of the carboxy terminal tail of the chicken gonadotropin-releasing hormone receptor was determined by testing the activity of a series of chicken gonadotropin-releasing hormone receptors with progressive deletions in their carboxyl terminus. The 55 amino acid carboxy terminal tail of the chicken gonadotropin-releasing hormone receptor was progressively truncated, resulting in cS320STOP, cR330STOP, cS337STOP, cS346STOP, cT351STOP, cD356STOP, cS366STOP and cC375STOP truncated mutants, which were all tested in parallel with the wild type chicken gonadotropin-releasing hormone receptor.

Truncation of the entire carboxy terminal tail from the chicken gonadotropin-releasing hormone receptor, cS320STOP abolished gonadotropin-releasing hormone binding and gonadotropin-releasing hormone-induced inositol phosphate production. The loss of gonadotropin-releasing hormone binding by the cS320STOP-truncated mutant suggests that this receptor is possibly not expressed on the cell membrane, which might be due to improper receptor folding by cS320STOP. The carboxy terminal tail of the chicken gonadotropin-releasing hormone receptor might therefore be required for proper folding of newly formed chicken gonadotropin-releasing hormone receptors and expression of these receptors on the cell membrane. The cR330STOP mutant had a maximal gonadotropin-releasing hormone binding of ~12%, which is the lowest receptor expression detected. The amino acid region between P^{319} and L^{329} might therefore play a role in receptor expression. Progressive increase in the carboxy terminal tail from L^{329} resulted in progressive increase in the receptor expression. Maximal gonadotropin-releasing hormone binding levels reached wild type levels at truncation of the cGnRHR at S^{366}. These results indicate that the first 45 amino region, ie. between P^{319} and S^{366} of the chicken gonadotropin-releasing hormone receptor carboxy terminal tail contains elements that promote receptor expression.

Gonadotropin-releasing hormone-induced inositol phosphate production was enhanced for all the truncated receptors except cR330STOP and cS337STOP, though all the truncated receptors had coupling efficiency values larger than the wild type chicken gonadotropin-releasing hormone receptor. This enhanced inositol phosphate production might be due to an increased coupling efficiency between the truncated chicken gonadotropin-releasing hormone receptors and the $\alpha_{q/11}$-type G-protein. However, none of the truncated chicken gonadotropin-releasing hormone receptors

have left-shifted EC_{50} values, indicating that coupling efficiency did not increase. Alternatively, a loss or retardation in receptor desensitization and/ or internalization for the truncated chicken gonadotropin-releasing hormone receptor mutants might be responsible for the enhanced gonadotropin-releasing hormone-induced inositol phosphate production by the truncated chicken gonadotropin-releasing hormone receptors.

The chicken gonadotropin-releasing hormone receptor has a highly conserved cysteine residue in position 328 that might be palmitoylated. Replacing this cysteine in the chicken gonadotropin-releasing hormone receptor with an alanine [cC328A] increased receptor expression 2 fold, reduced maximal inositol phosphate production to ~69% and severely impaired coupling efficiency to 30% relative to the wild type levels. This finding indicates that C^{328} might be palmitoylated and is required for receptor coupling.

In conclusion, the amino terminal region of the chicken gonadotropin-releasing hormone receptor carboxy terminal tail increases receptor expression, either by affecting the transport of newly synthesized chicken gonadotropin-releasing hormone receptors to the plasma membrane and/or the proper folding of this receptor. The intracellular carboxy terminal tail of the chicken gonadotropin-releasing hormone receptor might play a negative role in G-protein coupling. However, the enhanced inositol phosphate production from the truncated chicken gonadotropin-releasing hormone receptors could be due to reduced internalization and/ or desensitization of the carboxy terminal truncated receptors. Point-mutation of C^{328} to A resulted in decreased coupling suggesting that C^{328} may be a palmitoylation site and might play a role in coupling or desensitization.

2. INTRODUCTION

2.1 GENERAL INTRODUCTION

The gonadotropin-releasing hormone [GnRH] is a decapeptide, synthesised by enzymatic cleavage of a large precursor protein in neurons of the medial basal hypothalamus. It plays a major role in maintaining homeostasis of the neuro-endocrine reproductive axis. This hormone product is secreted via the hypophyseal blood circulation in a pulsatile manner to the anterior pituitary, where it binds with high affinity to the GnRH receptors [GnRHRs] expressed on the plasma membrane of gonadotropes. Upon binding of GnRH they mediate the synthesis and release of the gonadotropins, luteinizing hormone [LH] and follicle-stimulating hormone [FSH] which regulate gonadal steroidogenesis and gametogenesis. Early studies on regulation of LH release from gonadotropes showed that the pulse timing as well as the concentration of GnRH is critical for gonadotropin release (Lui and Jackson, 1984). Chronic doses of GnRH administration to gonadotropes, was found to have an inhibitory effect on gonadotropin secretion from gonadotropes in a number of different species, such as in sheep (McIntosh and McIntosh, 1985), chicken (King *et al.*, 1986) and rat (Hawes and Conn, 1992). This phenomenon was later exploited clinically in the treatment of pathophysiological disorders linked to the reproductive system, for example precocious puberty (Boepple *et al.*, 1986), breast cancer (Manni *et al.*, 1986), and prostate cancer (Labrie *et al.*, 1986). This revolutionised the research field in reproductive physiology and triggered the design and synthesis of many novel GnRH agonists and antagonists.

The primary amino acid sequence of mammalian GnRH was identified by Matsuo *et al.* (1971) who isolated this peptide from pig hypothalamus. Since then different forms of GnRH has been identified from both mammalian and non-mammalian species as seen in **figure 2.1** (King and Millar, 1995; Sealfon *et al.*, 1997). Most species have more than one form of GnRH. Mammalian GnRH controls the reproductive axis in mammals, while in chickens it is CI GnRH. The N- and C-terminal domains of GnRHs are highly conserved and are both required for binding to the GnRHR. The N-terminal domain is however more important for GnRHR activation (Sealfon *et al.*, 1997). GnRH is believed to take on a constrained form when it is in the active conformation. A glycine residue is conserved in position 6 of

believed to allow flexibility to the peptide during conformational transition between the active and inactive state (Sealfon *et al.*, 1997).

Mammal	E H W S Y G L R P G
Chicken I	E H W S Y G L Q P G
Seabream	E H W S Y G L S P G
Catfish	E H W S H G L N P G
Salmon	E H W S Y G W L P G
Dogfish	E H W S H G W L P G
Chicken II	E H W S H G W Y P G
Lamprey III	E H W S H D W K P G
Lamprey I	E H Y S L E W K P G
Tunicate I	E H W S D Y F K P G
Tunicate II	E H W S L C H A P G

Figure 2.1 Primary amino acid sequence of GnRH from mammalian and non-mammalian species. Highly conserved residues are indicated in red.

2.2 GnRHR SIGNALING

All G-protein coupled receptors [GPCRs] transduce signals via heterotrimeric GTP-binding proteins [G-proteins] that consist of a α, β- and γ-subunit. In the inactive state the α-subunit binds GDP and is associated with βγ, while in the active state the α-subunit is bound to GTP and is dissociated from the βγ-subunit. A number of G-proteins, including $G\alpha_s$, $G\alpha_o$, $G\alpha_i$, $G\alpha_q$, etc. have been identified by combining molecular cloning techniques with the inhibitory and stimulatory effects of bacterial toxins on specific G-protein classes (Neer, 1995). Early studies on gonadotrope stimulation with GnRH showed that activation of the GnRHR leads to an increased release in inositol phosphates [IPs] and calcium [Ca^{2+}] mobilization, which can occur in the presence of pertussis toxin [PTX] (Naor *et al.*, 1986). Stimulation of mouse gonadotrope [αT3-1] cell membranes in the presence of an antibody against PTX-insensitive $G\alpha_{q/11}$ resulted in a marked decrease in IP production, suggesting that the GnRHR couples to $G\alpha_{q/11}$ (Hsieh and Martin, 1992). Ligand stimulation of the

GnRHR couples to $G\alpha_{q/11}$ (Hsieh and Martin, 1992). Ligand stimulation of the GnRHR induces a conformational change in the receptor. The activated GnRHR in turn catalyses $G\alpha_{q/11}$ to exchange GDP for GTP, resulting in G-protein activation. The activated GTP-bound $G\alpha_{q/11}$ dissociates from the $\beta\gamma$ subunits and relays the message to β-inositide specific phospholipase C [PLC-β]. PLC-β in turn hydrolyses the membrane embedded phosphatidylinositol 4,5-bisphosphate [PIP_2] into second messengers inositol 1,4,5-trisphosphate [IP_3] and diacylglycerol [DAG]. The generated IP_3 and DAG promote the mobilization of Ca^{2+} from the endoplasmic reticulum [ER] stores and activates protein kinase C [PKC] respectively. This results in the propagation of a signaling cascade that culminates in the synthesis and exocytosis of LH and FSH from gonadotropes (Stojilkovic *et al.*, 1994).

Various GPCRs have been shown to mediate signals via more than one G-protein, for example the thyroid-stimulating hormone receptor [TSHR] (Allgeier *et al.*, 1994) and α_{2A}-adrenergic receptor [α_{2A}-AR] (Chabre *et al.*, 1994). Such promiscuity allows the activation of more than one signaling system by a single receptor type. Arora *et al.* (1998) and Ulloa-Aguirre *et al.* (1998) respectively, demonstrated that GnRH-induced activation of the mouse GnRHR [mGnRHR] and rat GnRHR [rGnRHR] results in increased cAMP production. Thus, in addition to transmitting signals via $G\alpha_{q/11}$, the GnRHR also couples to $G\alpha_s$. Furthermore, Stanislaus *et al.* (1998) suggests that the GnRHR might also couple to $G\alpha_{i/o}$. GnRH-induced signaling via $G\alpha_{q/11}$ and $G\alpha_s$ is, however, not only observed in mammalian GnRHRs, but also non-mammalian GnRHRs such as the catfish GnRHR [cfGnRHR] (Tensen *et al.*, 1997).

GnRHR activation results in stimulation of the mitogen-activated protein kinase [MAPK] and Jun N-terminal kinase [JNK] pathways via PKC (Sundaresan *et al.*, 1996; Nurel *et al.*, 1998). The MAPK and JNK signaling cascade each consist of serine/threonine kinases that phosphorylate transcription factors, resulting in trancriptional regulation of a variety of genes. Little is known about the role of the MAPK and JNK cascade in response to GnRH. However, there is evidence that these pathways activate transcription factors that are responsible for GnRH-regulated transcription of gonadotropins and the GnRHR (Roberson *et al.*, 1995; Saunders *et al.*, 1998; Nurel *et al.*, 1998). The initial transcriptional regulation in response to

GnRH induction is presumably governed mainly by the GnRH-MAPK pathway, followed by the GnRH-JNK pathway (Nurel *et al.*, 1998).

2.3 CLONING OF THE GnRH RECEPTOR

Research concerning the effects of GnRH on gonadotropes as well as identifying the molecular mechanism involved in this signaling event remained the focal point of reproductive research after the primary amino acid sequence of mammalian GnRH was elucidated (Matsuo *et al.*, 1971). However, research in this field reached a turning point when the cDNA of the mGnRHR was cloned. RNA extracted from αT3-1 cells was used to express a functional GnRHR in *Xenopus* oocytes, which led to the construction of cDNA library of αT3-1 cells. These cDNA libraries were used to generate full-length cDNA clones of the mGnRHR by means of PCR, using degenerate oligonucleotides homologous to conserved transmembrane [TM] motifs of G-protein coupled receptors [GPCRs] (Tsutsumi *et al.*, 1992). Shortly thereafter, GnRHRs were cloned from other mammalian species, for example rat (Eidne *et al.*, 1992), human (Chi *et al.*, 1993), and sheep (Illing *et al*, 1993; Brooks *et al.*, 1993). Alignment of their primary sequences revealed that these receptors share more than 85% amino acid homology (Davidson *et al.*, 1994). Hydrophobicity analysis showed that the GnRHR has seven putative TMs that are characteristic of GPCRs. These TMs are connected on the outside of the cell by three extracellular loops [ECLs] and on the inside by three intracellular loops [ICLs]. The most striking difference between the mammalian GnRHRs and other GPCRs is the absence of an intracellular C-terminal tail from mammalian GnRHRs, which is normally present in all GPCRs cloned [**figure 2.2**]. Recently, various non-mammalian GnRHRs were cloned, for example catfish (Tensen *et al.*, 1997), frog (Troskie B., unpublished), goldfish (Illing *et al.*, 1999) and chicken (Sun Y., unpublished), which revealed significant differences to their mammalian counterparts. One of the most notable differences is that the intracellular C-terminal tail is present in all non-mammalian GnRHRs cloned [**figure 2.3**]. The cloning of the GnRHR cDNAs from different species proves to be an invaluable tool, facilitating a closer look at the GnRHR in terms of its structure and the identification of domains important in ligand binding and G-protein coupling.

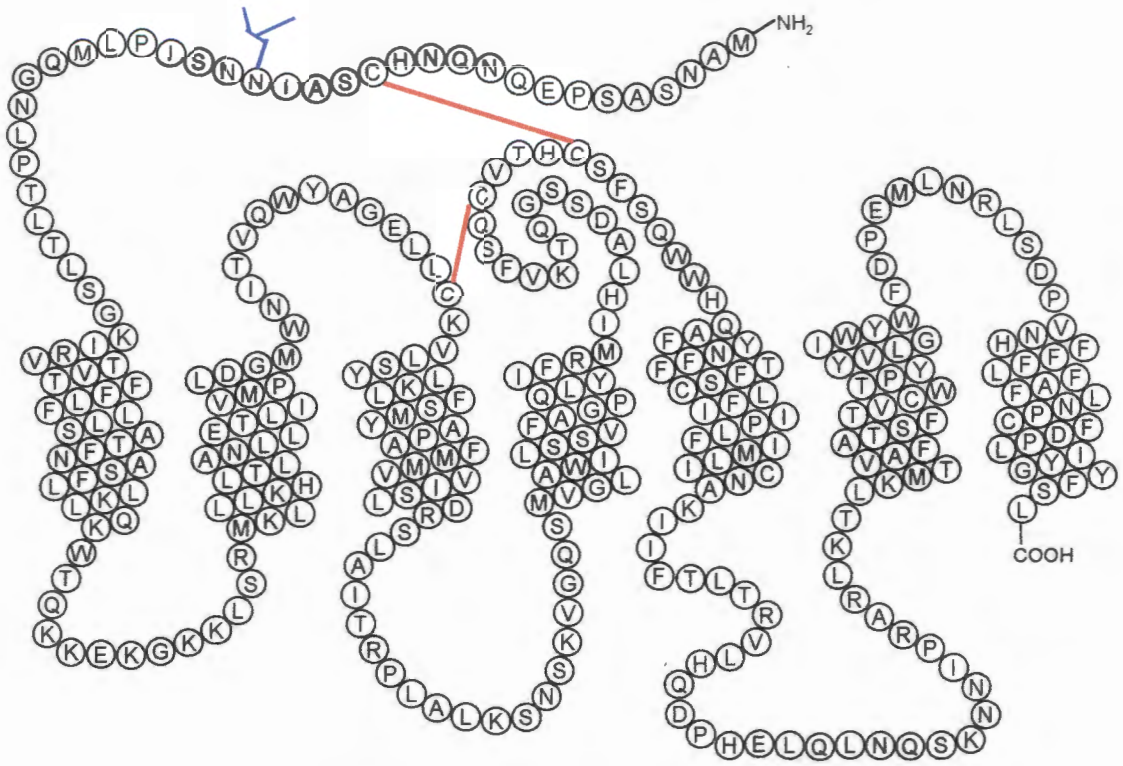


Figure 2.2 Diagram of the human GnRHR that lacks an intracellular C-terminal tail, an N-linked glycosylation site [blue] and two disulphide bonds [red] are indicated.

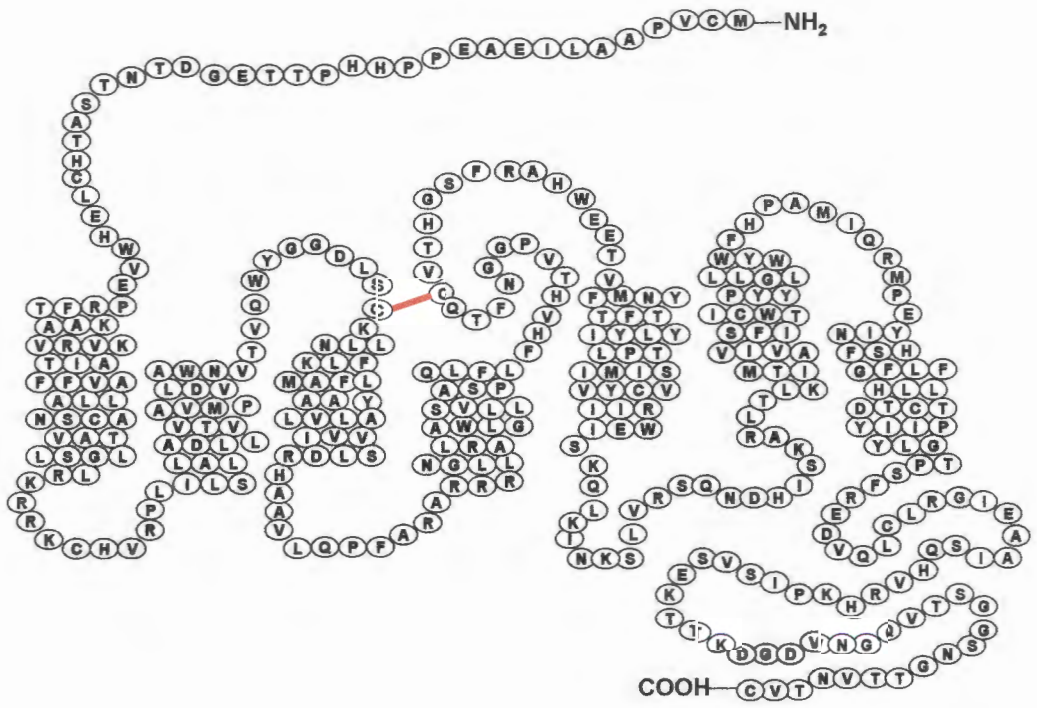


Figure 2.3. Diagram of the chicken GnRHR that possesses two conserved cysteine residues, one in TM3 and another in ECL2 that might possibly form a disulphide bond [red].

2.4 G-PROTEIN COUPLING OF THE GnRHR

The intracellular loop [ICL] domains are the only structural entities of the GPCRs protruding the intracellular milieu and therefore it is not surprising that these domains were shown to interact with the cytosolic G-proteins. Cooperativity between two or more ICL domains, including the C-terminal tail has been demonstrated to orchestrate receptor/G-protein coupling in various GPCRs, such as the M_3-R (Blin *et al.*, 1995), β_2-AR (O'Dowd *et al.*, 1988), AT_{1A}-R (Hunyudy *et al.*, 1994) and V_2-R (Erlenbach and Wess, 1998).

Arora *et al.* (1998) demonstrated that the first ICL of the mGnRHR possesses specific residues at the N- and C-terminus that are critical for $G\alpha_s$ activation, but not $G\alpha_{q/11}$. Replacing the N-terminal L^{58} and C-terminal L^{73} of ICL I with A and R respectively dramatically reduced GnRH-induced cAMP response by 80%. Furthermore, substitution of the C-terminal S^{74} and L^{80} with E and A respectively, completely abolished GnRHR-mediated cAMP production. These point mutations did not interfere with GnRH-induced IP signaling.

A highly conserved motif, DRYXXV/IXXPL [where X is any amino acid] at the N-terminus of ICL 2 in most GPCRs, is also present in the mammalian GnRHR at a homologous position with the exception of a serine residue replacing the putative tyrosine. Arora *et al.* (1995) reported that this specific serine residue is not involved in GnRHR/$G\alpha_{q/11}$ coupling. In contrast, mutation of D^{138} to N or E and substitution of R^{139} with Q significantly impaired GnRHR-mediated IP production. This suggests that these highly conserved residues play an important role in GnRHR/$G\alpha_{q/11}$ coupling or GnRHR activation (Arora *et al.*, 1997; Ballesteros *et al.*, 1998).

Substituting L^{147} of the mGnRHR in the D^{138}RSXXIXXPL^{147} motif with A or D, significantly reduced GnRH-induced IP production by more than 50 and 70% respectively. L^{147} is therefore involved in $G\alpha_{q/11}$ coupling of the GnRHR (Arora *et al.*, 1995). Interestingly, this hydrophobic residue is conserved in both mammalian and non-mammalian GnRHRs, except the chicken GnRHR [cGnRHR] that contains a larger hydrophobic phenylalanine instead of a leucine. A hydrophobic residue at this position might have a conserved role in coupling of the GnRHRs with $G\alpha_{q/11}$ (Moro *et al.*, 1993).

P^{146} of the human GnRHR [hGnRHR] is highly conserved in ICL 2 of both mammalian and non-mammalian GnRHRs. Coupling efficiency of the hGnRHR was

significantly reduced by substituting R^{145} with P, introducing a P-P motif (Sealfon *et al.*, 1997). These results suggest that P^{146} of the GnRHR may be responsible for orientating ICL 2, presenting specific G-protein coupling domains of this loop to the G-protein and/or other loop domains that it may co-operate with during G-protein coupling.

Ulloa-Aguirre *et al.* (1998) transiently expressed the third ICL of the rGnRHR into pituitary-derived lactotrope [GH3] cells stably transfected with the rGnRHR [GGH_31^1 cells]. This homologous co-expression inhibited Buserelin-induced IP response by 20% as well as cAMP production by 30%. Thus, the third ICL of the rGnRH receptor may be involved in $G\alpha_{q/11}$ and $G\alpha_s$ coupling in GGH_31^1 cells. Myburg *et al.* (1998) demonstrated that substitution of A^{261} in the hGnRHR with bulky amino acids, such as F, E, K, L, and I abolished ligand-induced IP response. This data suggest that A^{261}, which is localized in the C-terminal domain of the hGnRHR ICL 3, plays a critical role in the coupling of this receptor to $G\alpha_{q/11}$. Some of the residues required for coupling are likely to interact directly with the G-protein, while others appear to be important for the conformational change within the receptor induced by ligand binding.

2.5 THE ROLE OF THE INTRACELLULAR C-TERMINAL TAIL IN:

• EXPRESSION

Very little is known about the process in which newly synthesized GPCRs are folded and transported to the plasma membrane and which regions of these receptors are involved in receptor expression. Results from truncation and deletion mutations of the intracellular C-terminal tail from a number of different GPCRs suggest that this region contains certain amino acid residues that are involved in receptor expression. Partial truncation of the luteinizing hormone receptor [LHR] by 48 amino acids resulted in a marked reduction in receptor expression, which was less than half the level of the full-length LHR (Sánchez-Yagüe *et al.*, 1992). A similar effect was observed in other GPCRs, including the RhR (Weiss *et al.*, 1994) and parathyroid hormone receptor (Huang *et al.*, 1995). In contrast, truncation of 60 and 70 amino acids from the NK_2-R intracellular C-terminal tail resulted in a 3.3 and 1.4 fold

increased receptor expression respectively (Alblas *et al.*, 1995). This suggests that the LHR may possess positive regulatory elements in its C-terminal tail, as opposed to possible negative regulatory elements present in the C-terminal tail of the type 2 neurokinin receptor [NK_2-R] that play a role in receptor expression.

In a recent study, the addition of the cfGnRHR intracellular C-terminal tail to the rGnRHR resulted in a ~5 fold higher receptor expression of the chimera compared to the wt rGnRHR (Lin *et al.*, 1998). Similarly, when the intracellular C-terminal tail of the rTRHR was added to the rGnRHR, the chimeric rGnRHR/rTRHR-C-tail expression was increased 3.1 fold in comparison to that of the wt rGnRHR (Heding *et al.*, 1998).

Sadeghi, *et al.* (1997) showed that the naturally occurring C-terminal tail truncated mutant V_2R-R337stop [9 amino acid C-terminal tail length], present in patients suffering from X-linked recessive nephrogenic diabetes insipidus, is not expressed on the plasma membrane of African green monkey kidney [COS.M6] cells or HEK293 cells. However, they found that the V_2R-R337stop mutant is produced in similar quantities as the wt V_2R, but is only present in the immature Endoglycosidase H sensitive form. An additional length of 4 amino acids is required [V_2R-341stop] to yield a mature receptor form expressed on the cell surface that binds ligand and mediates arginine vasopressin stimulation. With the use of fluorescence microscopy Oksche *et al.* (1998) demonstrated that the V_2R-R337stop mutant receptor is retained in the endoplasmic reticulum [ER], possibly due to improper folding of the truncated receptor. This may implicate the membrane proximal region of the C-terminal tail in proper folding of the wt V_2R. Furthermore, Rozell *et al.* (1998) showed that immature forms of G-protein coupled LHR and follicle stimulating hormone receptor [FSHR] associate with the protein folding chaperone calnexin, which facilitates protein folding and prevents the migration of improperly folded proteins from the ER. Thus the improperly folded V_2R-R337stop mutant receptor may possibly be retained in the ER by protein folding chaperones. Oksche *et al.* (1998) suggested that the distal region of the C-terminal tail is required for efficient expression of the V_2R.

Using an *in vitro* retinal cell-free system, Deretic *et al.* (1996) demonstrated that addition of a monoclonal antibody directed against the C-terminal tail of the Rh-R significantly inhibited post-Golgi vesicle formation. This indicates that the C-terminal tail of the Rh-R may interact with proteins or factors involved in mediating post-Golgi vesicle formation, thus playing a role in protein sorting.

All these findings suggest that the intracellular C-terminal tail of GPCRs may be involved in the proper folding of the receptor and or transport of the receptor to the plasma membrane in which the C-terminal tail of the cGnRHR may play a role. The mammalian GnRHRs lack an intracellular C-terminal tail, yet they are expressed on the plasma membrane and are functional. In contrast the non-mammalian GnRHRs, like most GPCRs have an intracellular C-terminal tail. It would therefore be interesting to examine the role of the C-terminal tail of the cGnRHR in the receptor expression and activity.

• COUPLING

The intracellular C-terminal tail of several GPCRs has been shown to be important in G-protein coupling. For example the intracellular C-terminus of the type 1B α-adrenergic receptor [α_{1B}-AR] dramatically inhibited ligand-induced IP stimulation from the full-length α_{1B}-AR during homologous co-expression in human embryonic kidney [HEK-293] cells (Hawes *et al.*, 1994). The importance of the C-terminal tail in coupling was also demonstrated with the rhodopsin receptor [Rh-R] (Weiss *et al.*, 1994), follicle-stimulating hormone receptor [FSHR] (Grasso *et al*, 1995), interleukin-8 receptor (Ben-Baruch *et al.*, 1998), and calcitonin receptor (Findlay *et al.*, 1994).

In a gain-of-function study the intracellular C-terminal tail of the IP-linked type 1a vasopressin receptor [V_{1a}-R] was replaced with that of the cAMP-linked type 2-vasopressin receptor [V_2-R]. The resultant vasopressin type 2 and 1a chimeric receptor gained a 28% $G\alpha_s$ coupling ability, while reducing the maximal IP production by ~20% (Erlenbach and Wess, 1998). Hence, the intracellular C-terminal tail appears to be involved in receptor/G-protein coupling of both IP- and cAMP-linked GPCRs.

Amongst other regions in the human β_2-AR, the N-terminal portion of its intracellular C-terminal tail shares a high homology with other GPCRs. These include the β-AR subtype 1 and 2 from different species, the human muscarinic acetylcholine receptor subtype 1, 2, 3 and 4; as well as the human Rh-R. Therefore specific residues in the N-terminal region of the human β_2-AR C-terminal tail were substituted and deleted to establish if the N-terminal portion is important in G-protein coupling. This resulted in decreased maximal adenylyl cyclase activation suggesting that the N-terminal region of the C-terminal tail plays a role in β_2-AR coupling to G_s (O'Dowd *et al.*, 1988). A

similar experiment was performed in the α_1-AR which activates the phospholipase C second messenger system. Thirteen amino acids in the N-terminal region of the α_1-AR intracellular C-terminal tail were replaced with that of the β_2-AR. The resultant chimera produced a decreased maximal IP activity without any change in receptor expression or binding affinity (Cotecchia *et al.*, 1990). Thus, the N-terminal domain of the C-terminal tail appears to be important in receptor/G-protein coupling of cAMP- and IP-linked GPCRs.

The N-terminal domain of the C-terminal tail of a number of GPCRs possess one or more conserved cysteine residue that is palmitoylated for example in the type A and B endothelin receptors (Horstmeyer *et al.*, 1996; Okamoto *et al.*, 1997). Palmitoylation of the C-terminal tail of GPCRs is believed to form a fourth loop by anchoring the C-terminal tail into the membrane. Alanine replacement of a cluster of five cysteine residues in the membrane proximal domain of the type A endothelin receptor [ET_A-R] C-terminal tail abolished palmitoylation. The $G\alpha_s$ coupling of this unpalmitoylated mutant receptor remained unaffected, while the $G\alpha_q$ coupling was severely reduced (Horstmeyer *et al.*, 1996). In contrast, abolishing palmitoylation of the type B endothelin receptor [ET_B-R] did not affect $G\alpha_q$ coupling, but impaired $G\alpha_i$ coupling. Thus, palmitoylation appears to play a role in receptor/G-protein coupling.

Very little is known about the role of the intracellular C-terminal tail in coupling of the non-mammalian GnRHRs to their cognate G-proteins. Recently the 51 amino acid intracellular C-terminal tail of the cfGnRHR was truncated (Lin *et al.*, 1998). In contrast to the wt cfGnRHR that produces IPs in a dose-dependent manner in response to Buserelin stimulation, the truncated cfGnRHR mutant did not activate IP production. Because no detectable Buserelin binding could be measured for the wt or mutant cfGnRHR, it was not clear if the truncation affected receptor expression. No direct conclusion can be made from these experiments about the role of the cfGnRHR C-terminal tail in G-protein coupling. Furthermore, these authors engineered a chimeric rGnRHR/cfGnRHR-C-tail consisting of the intracellular C-terminal tail of the cfGnRHR fused to the rGnRHR. This chimeric rGnRHR/cfGnRHR-C-tail had a higher Buserelin-induced IP response with a ~2 fold decreased EC_{50} compared to the wt rGnRHR. This increased potency was not consistent with the ~5 fold increase in receptor expression recorded for this chimeric receptor. One can therefore not deduce whether the cfGnRHR C-terminal tail plays an important role, if any, in receptor/G-

protein coupling. However, considering the vast amount of evidence implicating the intracellular C-terminal tail of the GPCRs in G-protein coupling, the possibility of a similar role in non-mammalian GnRHRs, for example the cGnRHR can not yet be excluded.

• DESENSITIZATION

The levels of gonadotropin secretion changes dramatically during the course of the estrus and menstrual cycle, where LH release peaks prior to ovulation and reaches a nadir shortly thereafter. This indicates that gonadotropin release must be under tight regulatory control. Furthermore, continuous stimulation of gonadotropes with high concentrations of GnRH leads to attenuation of gonadotropin secretion [gonadotrope desensitization] in various species, including mammalians (Liu and Jackson, 1984; McIntosh and McIntosh, 1985) and non-mammalians (King *et al.*, 1986). Although the mechanism(s) involved in gonadotrope desensitization remain to be elucidated, this phenomenon proved invaluable in the clinical treatment of pathophysiological reproductive disorders, where patients were administered with chronic doses of GnRH (Manni *et al.*, 1986; Labrie *et al.*, 1986).

Desensitization commonly plays a role in GPCR regulation, where agonist-induced activation of a GPCR leads to phosphorylation of the activated receptor via G-protein coupled receptor kinases [GRKs] (Inglese *et al.*, 1993; Kwatra *et al.*, 1993; Ishii *et al.*, 1994). Subsequently, arrestin binds to the phosphorylated regions of the receptor, preventing receptor/G-protein coupling. This uncoupling event is known as homologous desensitization and it is responsible for mediating rapid desensitization. Unstimulated GPCRs can also undergo desensitization, known as heterologous desensitization, but phosphorylation of these receptors is governed by second-messenger-dependent kinases, ie. protein kinase A and C. Receptor internalization and down-regulation are responsible for mediating intermediate- and long-term desensitization effects respectively (Böhm *et al.*, 1997). Collectively these processes ensure signal termination.

Most early investigations on rapid homologous desensitization of GPCRs were performed on the cAMP-linked β-AR and cGMP-linked rhodopsin receptor. The intracellular C-terminal tail of both these receptors was implicated in mediating rapid homologous desensitization by phosphorylation of serine/threonine residues in their

intracellular C-terminal tail (Savarese and Fraser, 1992). However the α-adrenergic receptor undergoes rapid homologous desensitization due to phosphorylation of serine residues in its third ICL (Eason *et al.*, 1995). Moffett *et al.* (1996) abolished palmitoylation of the β_2-AR C-terminal tail by replacing C^{341} with a glycine residue. This increased the basal levels of receptor phosphorylation in the C-terminal tail, resulting in an uncoupled β_2-AR. Thus palmitoylation of the C-terminal tail might play a role in desensitization of some GPCRs.

Cloning of the mammalian GnRHRs revealed these receptors to be the only GPCRs that lack an intracellular C-terminal tail. Therefore a great deal of curiosity was ignited as to whether the mammalian GnRHRs can desensitize rapidly. Shortly thereafter Davidson *et al.* (1994) provided evidence suggesting that the mGnRHR does not undergo rapid homologous desensitization. Davidson and co-workers demonstrated that GnRH-induced IP accumulation increases linearly over 10min in αT3-1 cells and in GH_3 cells transfected with the mGnRHR. The thyroid releasing hormone receptor [TRHR], however displayed desensitization within 5min of stimulation in GH_3 cells (Davidson *et al.*, 1994). This is in accord with results published by Hawes *et al.* (1992), demonstrating that gonadotrope desensitization in response to GnRH is downstream to IP production.

Furthermore, Anderson *et al.* (1995) demonstrated that the GnRH-induced Ca^{2+} response attenuated during intermittent GnRH pulses [5min pulses at 15min intervals] in both pituitary [αT3-1 cells] and non-pituitary HEK293 cells [stably transfected with the rGnRHR]. This attenuated Ca^{2+} response is not a function of Ca^{2+} pool depletion because GnRH-releasable Ca^{2+} pool refilling occurs in less than 1min (McArdle *et al.*, 1996). This suggests that the desensitization might be at the Ca^{2+} mobilization level. In contrast, Willars *et al.* (1998) showed that αT3-1 cells pretreated with 100nM GnRH for 5min were equally robust in mediating a GnRH-induced Ca^{2+} response as non-pretreated αT3-1 cells. Thus, the comparable GnRH-induced Ca^{2+} mobilization from pretreated and non-pretreated αT3-1 cells demonstrates that the GnRHR does not desensitize rapidly.

The αT3-1 cell line does not lack the necessary machinery required for rapid desensitization, because other $G\alpha_q$-coupled GPCRs expressed in αT3-1 cells undergo rapid homologous desensitization of both IP and Ca^{2+} response (Willars *et al.*, 1998; Forrest-Owen *et al.*, 1999). This is hardly surprising because the αT3-1 cell line was

previously reported to express GRKs homologous to the rat GRK2, GRK3 and human GRK6 (Neill *et al.*, 1996). Forrest-Owen and co-workers also showed that the lack of rapid homologous desensitization of the mGnRHR in αT3-1 cells is not due the presence of "spare receptors". This is consistent with results previously published by Hawes *et al.* (1992), showing that homologous gonadotrope desensitization is not linked to the GnRHR number. To date, no conclusive evidence exists to support rapid homologous desensitization in the mammalian GnRHRs as seen in other GPCRs.

Recently, Heding *et al.* (1998) added the intracellular C-terminal tail of the rapidly desensitizing rat TRHR [rTRHR] to the rGnRHR, generating the rGnRHR/rTRHR-C-tail chimera. This chimera was able to desensitize rapidly in response to GnRH in both COS-7 [after 10min] and HEK293 [within 2min] cells, presumably via the rTRHR C-terminal tail. In contrast the wt. rGnRHR did not display rapid homologous desensitization. In addition, they also demonstrated that the cfGnRHR that does possess an intracellular C-terminal tail was able to undergo rapid homologous desensitization in COS-7 cells after about 5min and in HEK293 cells within 100sec. Willars *et al.* (1999) demonstrated that the cfGnRHR undergoes agonist-mediated phosphorylation, but not wt rGnRHR. Addition of the cfGnRHR C-terminal tail to the rGnRHR, resulted in a chimera [rGnRHR/cfGnRHR-C-tail] that undergoes agonist-mediated phosphorylation. This suggests that the cfGnRHR is probably phosphorylated at its C-terminal tail in response to agonist stimulation. The rGnRHR/cfGnRHR-C-tail chimera desensitized rapidly, unlike the wt rGnRHR counterpart. It is therefore possible that the cfGnRHR undergoes rapid homologous desensitization via its intracellular C-terminal tail, and that the lack of rapid desensitization in mammalian GnRHRs might be due to the absence of an intracellular C-terminal tail.

Both the chicken and catfish GnRHRs internalize rapidly in response to GnRHR stimulation (Pawson *et al.*, 1998; Heding *et al.*, 1998). Pawson *et al.* (1998) showed that truncation of the intracellular C-terminal tail of the cGnRHR reduced the rate of agonist-induced cGnRHR internalization from 11.3% per min to 0.55% per min. Thus, the C-terminal tail truncated cGnRHR mutant had an agonist-induced internalization rate similar to the hGnRHR [0.71% per min], implicating the C-terminal tail of the cGnRHR in mediating rapid internalization.

It therefore appears as though non-mammalian GnRHRs, for example the cGnRHR undergo rapid internalization and probably rapid desensitization via their intracellular C-terminal tail. These desensitization processes do not occur in mammalian GnRHRs and might be because of the evolutionary loss of their C-terminal tail.

3. MATERIALS AND METHODS

3.1 PCR MUTAGENESIS

Table 3.1: Primers used to truncate the cGnRHR and mutate C^{328} to alanine.

CONSTRUCT PRIMER	PRIMER SEQUENCE
cC375STOP*	5' GCACTCGAG*CTA*CACCGTGTTAACGGTTGTCC 3'
cS366STOP*[a]	5' GTCACTCGAG*TCA*GCCACCTGATGTCACCT 3'
cD356STOP*[a]	5' GTCACTCGAG*TCA*CCCATCCTTGGTGGTCT 3'
cT351STOP*[a]	5' GTCACTCGAG*TCA*CTTCTCTGAGACTGAGATG 3'
cS346STOP*[a]	5' GTCACTCGAG*TCA*GATGGGTTTGTGTCTCAC 3'
cS337STOP*	5' GTCACTCGAG*TCA*AATGGCTGCTTCAATGCCC 3'
cR330STOP*	5' GACCTCGAG*TCA*GAGACACAGTTGCACGTCC 3'
cS320STOP*	5' GTCACTCGAG*TCA*GGGGGTGTACAGTCCAT 3'
cC328A*	5' GGACTGTACACCCCAAGCTTTCGGGAGGACGT GCAACTGGCTCTCAGGGGCATTGAAGC 3'

- All the primers used for truncation of the cGnRHR C-terminal tail contain an *Xho I* site underlined and a stop codon written in italics.
- [a] marked primers were designed by Dr A. Pawson who also made their corresponding intracellular C-terminally truncated cGnRHR constructs.
- The cC328A* primer contains a *BsrG I* restriction site written in blue and a *Hind III* restriction site [silent mutation] indicated in green. Included in red is the alanine encoding sequence replacing the wt cysteine codon.

3.1.a Construction of Intracellular C-terminal truncated mutants

A series of C-terminal tail truncations of the cGnRHR were generated using specific antisense primers [listed in **table 3.1**] containing an *Xho I* restriction site and a stop codon. Each primer was used in combination with pcDNA I/Amp vector specific T7 sense primer [5' TAATACGACTCACTATAGGG 3'] and cGnRHR cDNA template [cloned into pcDNA I/Amp vector (Invitrogen) between *Not I* and *Xho I*] to generate PCR fragments encoding cGnRHRs with variable lengths of its intracellular C-terminus. Each PCR was catalysed by high fidelity Deep *Vent* polymerase (GIBCO) performed under the following conditions:

1min at 94°C [denaturation of cGnRHR/vector cDNA template]

1min at 50°C [annealing of primers to single stranded cDNA]

1min at 72°C [elongation of primers into new mutated cDNA strands], where this cycle was repeated 25-30 times; followed by 10min at 72°C to complete extension. The cGnRHR cDNA in pcDNA I/Amp has only one *Ava I* restriction site at base pair 942 of the insert. As depicted in **figure 3.A**, each PCR product was digested with restriction enzymes, *Ava I* (NEB) and *Xho I* (NEB), followed by isolation of the PCR fragment containing both *Ava I* and *Xho I* sticky ends from an 1.5% agarose gel. This fragment was purified using the QIAquick spin purification kit (QIAGEN) and ligated into pcDNA I/Amp vector construct containing wt cGnRHR cDNA that was linearised by *Ava I* and *Xho I*. The ligated construct was transformed into DH10B *E. coli* and grown on ampicillin agar plates. Positive clones were identified by restriction digest with *Not I* (NEB) and *Xho I*. Mutation of each truncated cGnRHR cDNA was confirmed by sequencing using the Sequenase Version 2.0 kit (USB).

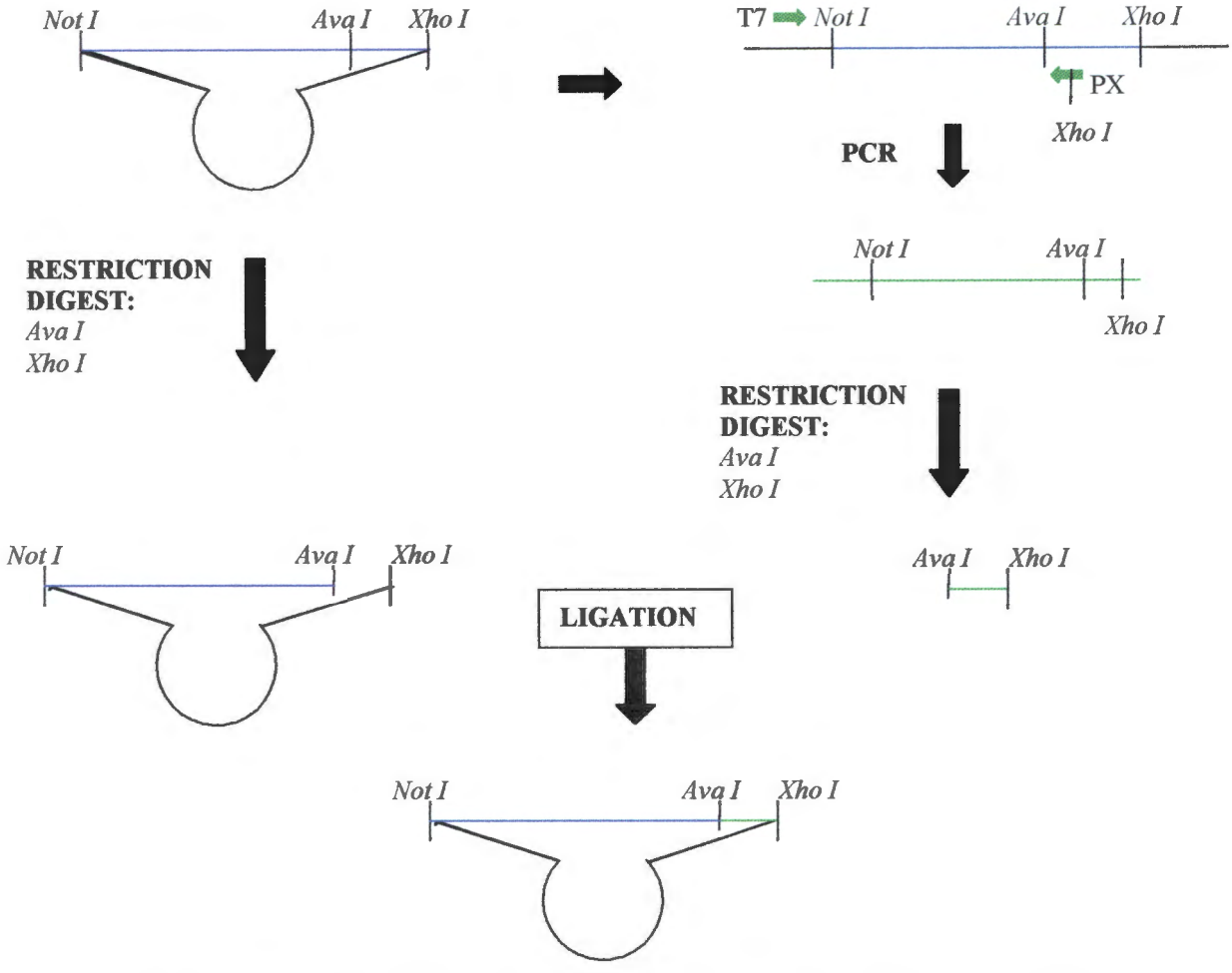


Figure 3.A Schematic representation of the method used to truncate the cGnRHR C-terminal tail by employing a PCR-based technique. Primer X [PX] represents any antisense primer given in **table 3.1** used to synthesise truncated forms of the cGnRHR. The vector, pcDNA I/Amp is depicted in black, the cGnRHR cDNA in blue, the primers and PCR fragment of the cGnRHR in green. See text for details.

3.1.b Construction of point-mutation of C^{328} to A

The sense cGnRHR primer cC328A* [**table 3.1**] contains an alanine encoding sequence [GCT] replacing the cysteine 328 encoding sequence [TGT], as well as a *Hind III* [silent mutation] and *BsrG I* restriction site. This primer in combination with the pcDNA I/Amp vector specific antisense Sp6 primer [5' GCATTTAGGTGACACTATA 3'] was used to amplify up the cGnRHR C-terminal tail encoding cDNA sequence under the same PCR conditions mentioned in **section 3.1a**. The cGnRHR cDNA has only one *BsrG I* restriction site at base pair 1014 of the insert. As described in **figure 3.B**, the A^{328} encoding PCR product was digested with restriction enzymes *BsrG I* (NEB) and *Xho I*, followed by isolation and purification. The purified fragment containing *BsrG I* and *Xho I* sticky ends was ligated into pcDNA I/Amp vector construct containing cGnRHR cDNA that was linearised by *BsrG I* and *Xho I*. DH10B *E. coli* cells were transformed with the ligated construct and grown on ampicillin agar plates. Ampicillin resistant clones were screened for *Hind III* (NEB) digestion and the positive clones sequenced in order to confirm the C^{328} to A mutation.

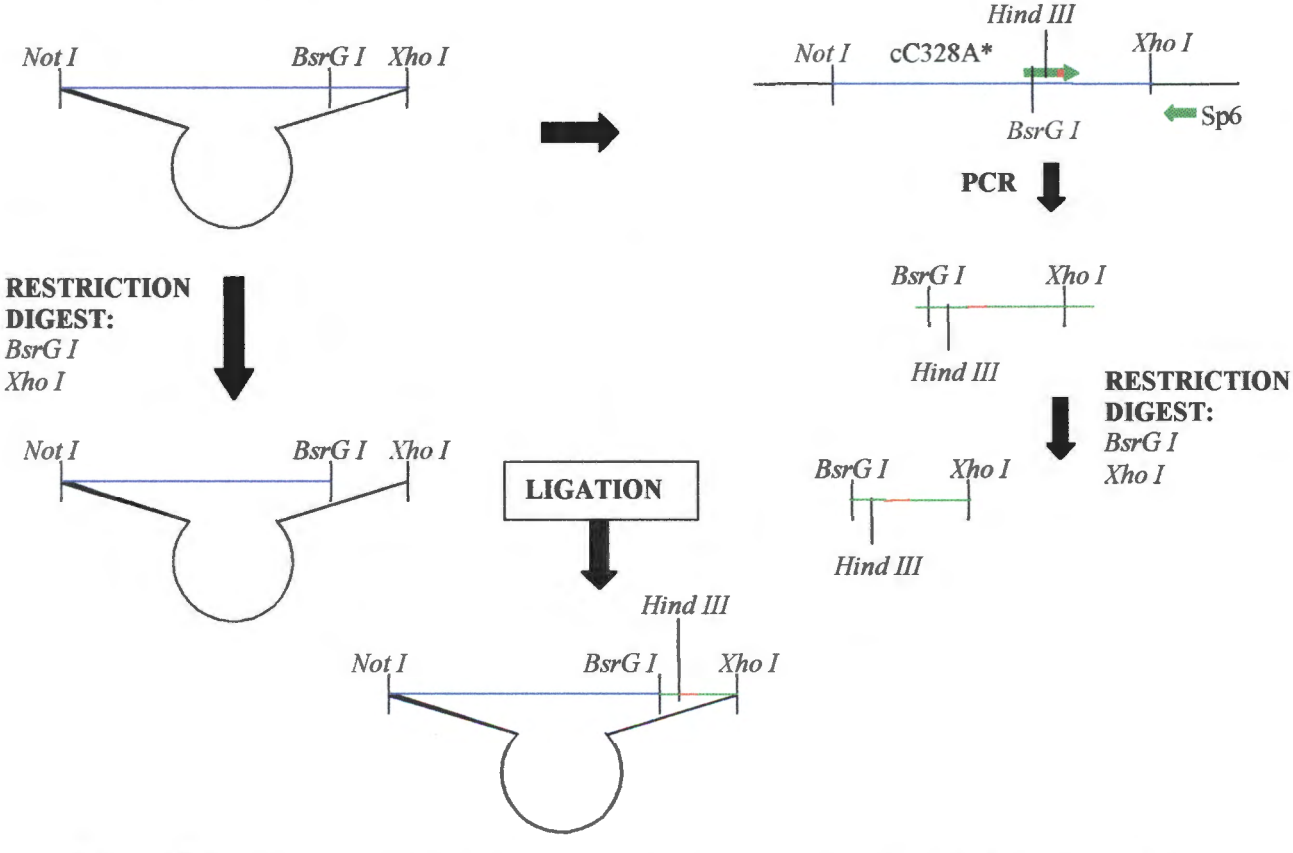


Figure 3.B Schematic representation of the strategy used to replace C^{328} in the cGnRHR with an alanine. The sense primer cC328A* has a silent *Hind III* restriction site and an alanine encoding sequence depicted in red. The vector, pcDNA I/Amp is depicted in black, the cGnRHR cDNA in blue, the primers and PCR fragment of the cGnRHR in green. See the text for details.

3.2 CELL CULTURING AND TRANSIENT TRANSFECTION WITH DEAE-DEXTRAN

COS-1 cells were maintained in low glucose DMEM media containing 10% heat inactivated fetal calf serum [FCS] at 37°C and 10% CO_2. For all transient transfections COS-1 cells were seeded on 12-well plates coated with poly-D-lysine. 250000 cells were seeded per well on 12-well plates and cultured overnight in 1ml/well of low glucose DMEM [10% FCS; 0.25% PS (2g/1000ml streptomycin sulphate; 2x 10^6U sodium benzylpenicillin)] at 37°C and 10% CO_2. DNA to be transfected [2.5µg/well] and 0.15mg DEAE-Dextran in HEPES-buffered saline (137mM NaCl; 5m MKCl; 0.7mM NaH_2PO_4; 21mM HEPES [pH 7.1]) were added to low glucose HEPES-DMEM containing 0.25% PS, making up a volume of 500µl/well. The final mixture was added to the cells. Cells were incubated for 4h at 37°C and thereafter washed once with low glucose HEPES-DMEM containing 0.25% PS. 1ml/well of 200µM chloroquine in low glucose DMEM [2% FCS; 0.25% PS] was added to the cells and removed after 1h incubation at 37°C, followed by incubation with 0.5ml/well of low glucose DMEM [10% DMSO; 0.25% PS] for 90sec. Thereafter the cells were washed once with low glucose HEPES-DMEM [0.25% PS], and fed with 1ml/well of low glucose DMEM [10% FCS; 0.25% PS].

3.3 WHOLE CELL BINDING ASSAY

A day after transfection the media of the cells were changed and the cells cultured overnight. The following day the cells were washed with 1ml/well of Buffer I (140mM NaCl; 4mM KCl; 20mM acid free HEPES; 8.3mM glucose; 1.5ml/l of 0.4% phenol red [pH 7.4]) containing 0.1% fatty acid free BSA; 1mM $MgCl_2$; 1mM $CaCl_2$. The cells were then incubated for 6h at 4°C with 80000cpm/well of [^{125}I]His^5-D-Tyr^6(GnRH) made-up in Buffer I containing 0.1% fatty acid free BSA; 1mM $MgCl_2$; 1mM $CaCl_2$ in a total volume of 600µl/well. This was done in the presence of increasing concentrations [0M; 10^{-11}M; 10^{-10}M; 10^{-9}M; 10^{-8}M; 10^{-7}M] of cold His^5-D-Tyr^6(GnRH) to compete with the radiolabeled [^{125}I]His^5-D-Tyr^6(GnRH) for receptor binding and each concentration was tested in duplicate. In order to determine non-specific binding, mock-transfected cells were incubated with 600µl of the radioactive binding solution in the absence of cold His^5-D-Tyr^6(GnRH). The radiolabeled media

was removed, cells were washed twice with 1ml/well of Buffer I containing 0.5% fatty acid free BSA; 1mM $MgCl_2$; 1mM $CaCl_2$ and then the cells were solubilised with 0.5ml/well of 0.1M NaOH. After shaking the plates for 5min, the radioactivity in each well was determined in a Gamma counter.

3.4 INOSITOL-PHOSPHATE ASSAY

One day after transfection the cells were washed once with Medium 199 containing 2% FCS; 0.25% PS and then incubated in this medium supplement with 1.5μCi/well of [^{3}H]myo-inositol. The cells were incubated overnight at 37°C, allowing at least 18h for label incorporation by the cells before the media was removed. The cells were washed twice with 1ml/well pre-warmed Buffer I [at 37°C] containing 0.1% fatty acid free BSA; 1mM $MgCl_2$; 1mM $CaCl_2$ with each wash lasting ~5min. The washing buffer was removed and the cells incubated for 1h at 37°C with increasing doses of GnRH ligand, 0M; 10^{-10}M; 10^{-9}M; 10^{-8}M; 10^{-7}M; 10^{-6}M. The chicken [CI] GnRH was made-up in Buffer I containing 1mM $MgCl_2$; 1mM $CaCl_2$ and 10mM LiCl. The ligand solutions were pre-warmed to 37°C and each concentration was tested in duplicate. After one-hour stimulation the media was removed and replaced with 1ml of ice cold 10mM formic acid, followed by incubation at 4°C for 30min. Dowex-1 columns [1 column/sample] were equilibrated with 3ml/column 3M ammonium formate, washed with 10ml/column distilled water and the samples were loaded onto the columns. The samples were washed with 10ml/column distilled water, followed by 5ml/column of 5mM myo-inositol containing 0,1M formic acid. The inositol phosphates were eluted from the columns with 3ml/column of 1M ammonium formate containing 0.1M formic acid, into 14ml/tube of Quicksafe Scintillation liquid (Zinsser Analytical) and the radioactivity counted on a beta counter.

3.5 CALCULATIONS OF RELATIVE COUPLING EFFICIENCY

The equation used for calculating the relative coupling efficiency values was developed by Ballesteros *et al.* (1998) in which the equation was derived as follows:

$[AR]/B_{max} = 1/(1 + K_d/[A])$ Equation 1

$E/E_{max} = 1/(1 + EC_{50}/[A])$ Equation 2

$Q = E/[AR]$ Equation 3

Substitution of equation 1 and 2 into 3 yields equation 4.

$Q = (1/2) \times [(K_d + EC_{50})/EC_{50}] \times (E_{max}/B_{max})$ Equation 4

$\sim Q = Q_{mutant}/Q_{wild\ type}$

where,

[AR] = concentration of ligand-receptor complex

[A] = concentration of unbound ligand

E_{max} = maximal receptor activation

E = EC_{50}

K_d = IC_{50} [this is under conditions where excess radiolabelled ligand is used to saturate the receptors expressed on the plasma membrane]

B_{max} = maximal receptor binding

Q = magnitude of receptor response per agonist-occupied receptor in arbitrary Numbers [efficiency of coupling]

~Q = coupling efficiency relative to the wild type

4. RESULTS

• THE ROLE OF THE INTRACELLULAR C-TERMINAL TAIL IN RECEPTOR EXPRESSION.

In order to determine whether the intracellular C-terminal tail of the cGnRHR is involved in the expression of this receptor, we initially deleted the entire intracellular C-terminal tail. A receptor mutant cS320STOP, lacking the C-terminal tail amino acids F^{321}-C^{375} was constructed in pcDNA I/Amp expression vector and transfected into COS-1 cells. No binding or GnRH-induced IP production could be detected for this mutant, suggesting that cS320STOP may not be expressed on the plasma membrane [data not showed]. As control, the wt cGnRHR was also tested, resulting in cGnRHR expression and a GnRH-induced IP response [**figure 4.1A** and **4.2A**]. The maximal Binding [B_{max}] and IP [IP_{max}] from the wt cGnRHR was taken as 100%. This suggests that the intracellular C-terminal tail of the cGnRHR may be required for receptor expression. The intracellular C-terminal tail was then progressively truncated to examine whether expression of the cGnRHR is dependent on a specific sequence contained in the intracellular C-terminal tail and/or the length of the intracellular C-terminal tail. As seen in **figure 4.1A** and **table 4.1**, cC375STOP and cS366STOP did not have a significantly reduced receptor expression. This indicates that the C-terminal region of the C-terminal tail from G^{365} to C^{375} does not play an important role in expression of the cGnRHR. Further truncations significantly reduced receptor expression, where progressive shortening of the C-terminal tail resulted in progressive reduction of receptor expression. A dramatic reduction of 55% expression can be seen with truncation of the C-terminal tail at D^{356} [cD356STOP] compared to the full-length cGnRHR. This suggests that the region between G^{355} and S^{366} may be important in receptor expression. No further reduction in expression of the cGnRHR is seen with truncation of the C-terminal tail at T^{351} and S^{346}. Truncation of the C-terminal tail at S^{337} and R^{330} resulted in a further decrease in expression to 30% and 12% respectively. The lowest expression level was displayed by the cR330STOP mutant. This truncated mutant receptor is 10 amino acids longer than the unexpressed cS320STOP mutant that lacks the entire C-terminal tail. These results suggest that the C-terminal tail of the cGnRHR is important for the expression of this receptor. **Figure 4.1B** and **table 4.1** shows that the affinities of the intracellular C-terminal truncated

cGnRHR mutants are all in the nanomolar range and are relatively comparable to that of the wt cGnRHR. This suggests that these truncated mutant receptors retained the wt conformation and that the C-terminal tail is not involved in ligand binding.

Table 4.1
Homologous competition binding assays were performed on the full-length cGnRHR and its truncated counterparts, using cold His^5-D-Tyr^6(GnRH) to compete against the [^{125}I]His^5-D-Tyr^6(GnRH) tracer for receptor binding. The maximal binding capacity [B_{max}] for each receptor was determined in the absence of cold His^5-D-Tyr^6(GnRH) and is given in percentage. The IC_{50} values are given in nanomoles [nM]. Each experiment was done in duplicate and repeated at least three times.

CONSTRUCT	B_{max} (% of wt.)	IC_{50} values (nM)
wt. cGnRHR	**100**	**2.8 ± 1.4**
cC375STOP	**92 ± 2**	**2.5 ± 0.8**
cC366STOP	**77 ± 35**	**4.7 ± 1.0**
cD356STOP	**45 ± 27**	**5.2 ± 2.5**
cT351STOP	**55 ± 28**	**5.3 ± 2.4**
cS346STOP	**61 ± 28**	**3.7 ± 1.7**
cS337STOP	**30 ± 10**	**2.4 ± 1.6**
cR330STOP	**12 ± 9**	**2.0 ± 1.1**

Figure 4.1A
The maximal percentage [^{125}I]His^5-D-Tyr^6(GnRH) binding [B_{max}] to the full-length cGnRHR and its C-terminal truncated mutants, in the absence of cold His^5-D-Tyr^6(GnRH).

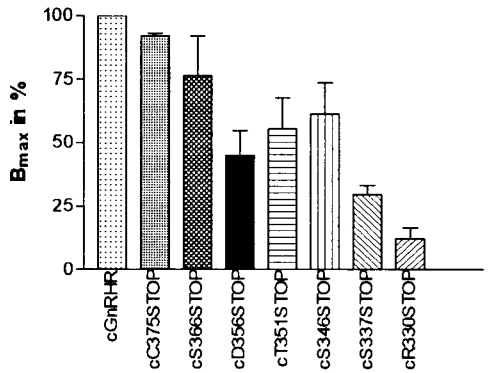
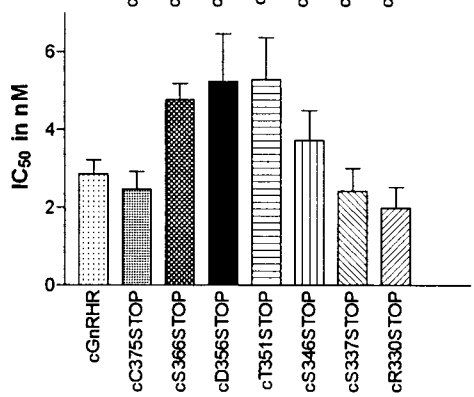


Figure 4.1B
IC_{50} values of the wt cGnRHR and the C-terminal truncated mutants, determined by competitive homologous His^5-D-Tyr^6(GnRH) binding, is given in nM.



• THE ROLE OF THE INTRACELLULAR C-TERMINAL TAIL IN RECEPTOR COUPLING.

The role of the cGnRHR C-terminal tail in coupling was determined by transfecting the truncated cGnRHR constructs into COS-1 cells and testing for their ability to generate IPs in response to CI GnRH. **Figure 4.2A** and **table 4.2** shows that cS337STOP and cR330STOP have a 60% and 24% maximal CI GnRH-induced IP production respectively, which is in correlation with their lower level of expression. The right-shifted EC_{50} values of these two truncated mutants is probably also on account of their low expression [**figure 4.2B and table 4.2**]. All the other truncated mutants with C-terminal tails longer than that of the cS337STOP mutant had a more robust maximal IP production than the full-length cGnRHR [**figure 4.2A** and **table 4.2**]. However, their EC_{50} values are comparable to that of the full-length cGnRHR [**figure 4.2B** and **table 4.2**]. The highest maximal IP response was generated by the cD356STOP mutant, which had almost a two fold greater IP yield than the wt. Yet, the wt cGnRHR had a two fold higher level of receptor expression than the cD356STOP mutant. Intuitively this truncated mutant should have a better coupling efficiency than the wt cGnRHR. This should also be the case for cS346STOP, cT351STOP, cS366STOP and possibly cC375STOP as they generate greater IP yields than the wt cGnRHR regardless of their lower expression levels. The relative coupling efficiency values were therefore determined for all the truncated cGnRHRs using the equation given in **methods 3.5** (Ballesteros *et al.*, 1998). Indeed, their relative coupling efficiency values are bigger than that of the full-length cGnRHR, implying that they couple more efficiently to $G\alpha_{q/11}$ [**table 4.3** and **figure 4.3**]. Unexpectedly, cS337STOP and cRR330STOP also had relative coupling efficiency values greater than the wt cGnRHR, 1.5 and 1.3 respectively. These results indicate that all the C-terminal truncated receptors couple to $G\alpha_{q/11}$ more efficiently than the full-length cGnRHR. Thus coupling of the cGnRHR is enhanced in the absence of a full-length C-terminal tail, suggesting that the C-terminal tail may play a negative regulatory role in $G\alpha_{q/11}$ coupling.

Table 4.2

A dose response of CI GnRH-induced IP production was determined for the full-length and truncated cGnRHRs. The maximal IP production [Ip_{max}] was determined using 10^{-6}M CI GnRH and is given in percentage. The EC_{50} values are given in nM. Each experiment was performed in duplicate and repeated at least three times.

CONSTRUCT	IP_{max} (% of wt.)	EC_{50} values (nM)
wt. cGnRHR	100	5.5 ± 4.1
cC375STOP	132 ± 16	4.1 ± 3.1
cS366STOP	147 ± 33	3.8 ± 3.7
cD356STOP	191 ± 53	9.1 ± 5.6
cT351STOP	158 ± 27	7.4 ± 5.2
cS346STOP	136 ± 27	2.9 ± 1.8
cS337STOP	60 ± 12	16.6 ± 6.5
cR330STOP	24 ± 6	109.9 ± 140.2

Figure 4.2A

Maximal GnRH-induced IP production [IP_{max}] of the wt and truncated cGnRHRs determined using 10^{-6}M CI GnRH and is given in %.

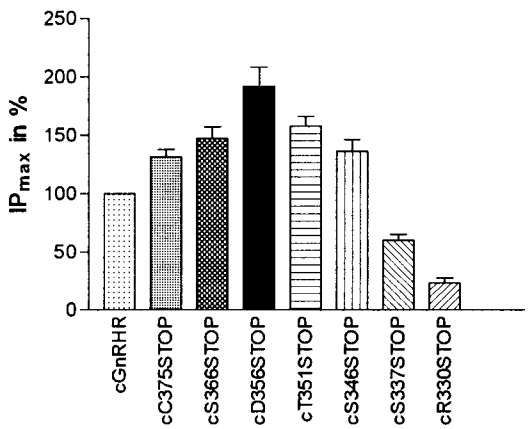


Figure 4.2B

EC_{50} values of the wt and truncated GnRHRs in nM. Determined from CI GnRH-induced IP dose-response.

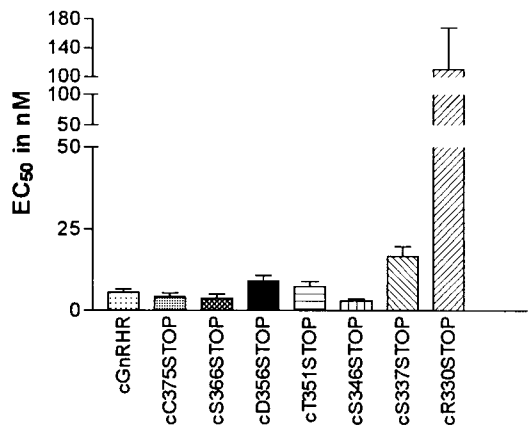
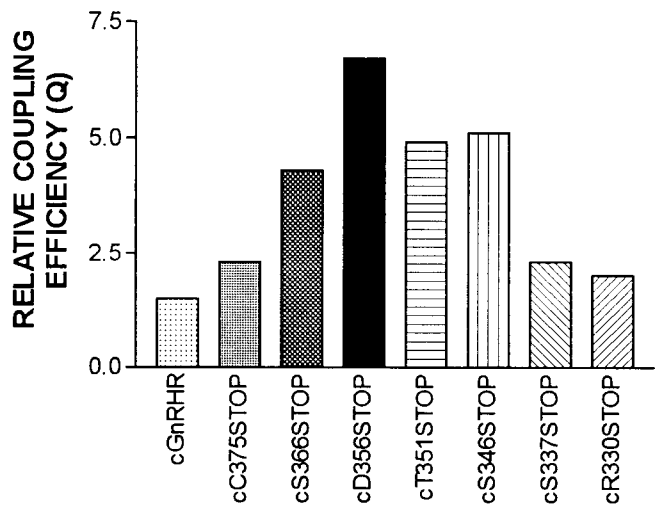


Table 4.3

Relative coupling efficiency [~Q] of the full-length and C-terminal truncated cGnRHRs, determined as explained in materials and methods **3.5,** are given in arbitrary numbers. Values larger than one suggests better coupling efficiency than the wt cGnRHR. Poorer coupling efficiency than the wt cGnRHR is indicated by values smaller than one.

CONSTRUCT	RELATIVE COUPLING EFFICIENCY [~Q]
cGnRHR	**1.0**
cC375STOP	**1.5**
cS366STOP	**2.9**
cD356STOP	**4.5**
cT351STOP	**3.3**
cS346STOP	**3.4**
cS337STOP	**1.5**
cR330STOP	**1.3**

Figure 4.3 Relative coupling efficiencies of the full-length cGnRHR and its C-terminal truncated counterparts.



• THE ROLE OF CYSTEINE 328 IN EXPRESSION AND COUPLING OF THE cGnRHR.

cGnRHR	^{320}S F R E D V Q L C^{328}
cfGnRHR	S F R A D L S R C
GfaGnRHR	S F R A D L A R C
GfbGnRHR	S F R A D I A S C

Figure 4.4 Alignment of the N-terminal domain of the chicken, catfish, goldfish A and B GnRHR C-terminal tails. The conserved cysteine residue is depicted in red.

Alignment of the membrane proximal C-terminal tail sequence of the cGnRHR with that of the cfGnRHR, goldfish type A and B GnRHR revealed a conserved cysteine residue 8 amino acids downstream to the intracellular TM7 boundary [**figure 4.4**]. Thus we surmised that C^{328} of the cGnRHR might possibly undergo palmitoylation, a modification present on cysteine residues in C-terminal tails of many GPCRs. Therefore, C^{328} of the cGnRHR was mutated to an alanine residue to investigate the role of this conserved residue in receptor coupling. **Figure 4.5A** and **table 4.4** shows that replacement of C^{328} with an alanine resulted in a two fold higher receptor expression level (204%) than the wt cGnRHR. The affinity of the cC328A mutant was 1.5 ± 0.3nM, which is similar to that of the wt cGnRHR, indicating that the cysteine to alanine mutation does not affect affinity [**figure 4.5B** and **table 4.4**]. A maximal IP yield of only 69% was produced by cC328A in response to CI GnRH [**figure 4.5C** and **table 4.4**], with an EC_{50} of 7.3 ± 5.5nM, which is similar to that of the wt cGnRHR [**figure 4.5D** and **table 4.4**]. This markedly reduced IP production from cC328A however, is in contrast to the two-fold increase in maximal binding measured for this mutant and suggests that coupling of this mutant receptor is decreased. The relative coupling efficiency determined for cC328A is 0.3, which is less than that of the wt cGnRHR [**figure 4.5E** and **table 4.4**]. Thus replacement of C^{328} with an alanine reduces the efficiency with which the cGnRHR couples to $G\alpha_{q/11}$, suggesting that C^{328} may play an important role in coupling of the cGnRHR. The cGnRHR has another cysteine residue in its C-terminal tail, C^{375}. This is the last residue in the C-

terminal tail and is not conserved amongst the non-mammalian GnRHRs. This implies that C^{375} might not play a major functional role and is probably not palmitoylated. Truncation of this residue [cC375STOP] did not affect expression or binding affinity [**table 4.1**, **figure 4.1A** and **figure 4.1B**]. The maximal IP production and relative coupling efficiency of cC375STOP however increased to 132% and 1.5 respectively [**table 4.2** and **table 4.3**]. No change in the EC_{50} was observed compared to the wt cGnRHR.

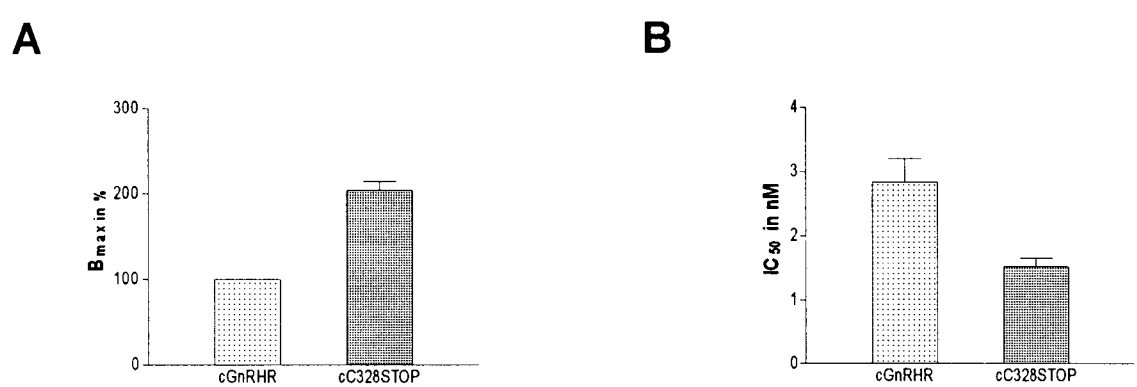
Table 4.4

Percentage maximal [^{125}I]His^5-D-Tyr^6(GnRH) binding [B_{max}] and IC_{50} [in nM] of the cC328A mutant receptor and wt cGnRHR, as determined from homologous competition binding. The percentage maximal IP production [IP_{max}] and EC_{50} [in nM] for the wt and cC328A mutant cGnRHR was determined from a CI GnRH-induced dose response. Each experiment was performed in duplicate and repeated at least three times. Relative coupling efficiency values were calculated as in materials and methods **3.5**, and are given in arbitrary numbers.

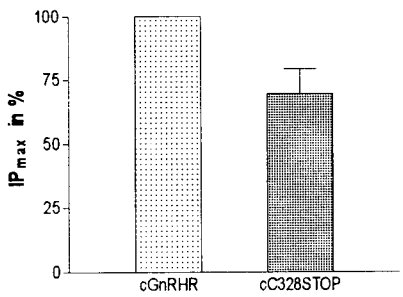
CONSTRUCT	B_{max}	IC_{50}	IP_{max}	EC_{50}	Relative Coupling Efficiency (~Q)
cGnRHR	**100**	**2.8 ± 1.4**	**100**	**5.5 ± 4.1**	**1.0**
cC328A	**204 ± 21**	**1.5 ± 0.3**	**69 ± 20**	**7.3 ± 5.5**	**0.3**

Figure 4.5

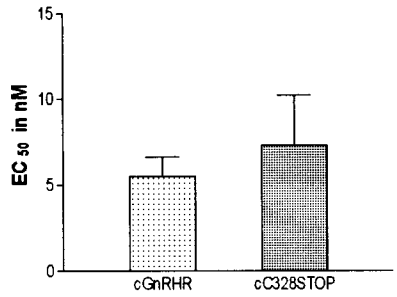
cC328A was compared to the wt cGnRHR in a homologous competition binding assay and a CI GnRH-stimulated dose response reaction. The maximum percentage [^{125}I]His^5-D-Tyr^6(GnRH) binding [B_{max}], determined in the absence of cold [^{125}I]His^5-D-Tyr^6(GnRH) is shown in **figure A**. The IC_{50} is given in nM and shown in **figure B**. The maximum percentage IP production [IP_{max}], determined using 10^{-6}M CI GnRH is shown in **figure C**. The EC_{50} values are given in nM and are shown in **figure D**. The calculated relative coupling efficiency of cC328A and the wt cGnRHR is depicted in **figure E**.



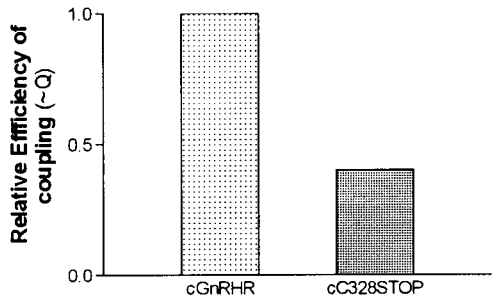
C


100
75
50
25
0
IP max in %
cGnRHR
cC328STOP

D


15
10
5
0
EC 50 in nM
cGnRHR
cC328STOP

E


1.0
0.5
0.0
Relative Efficiency of coupling (~Q)
cGnRHR
cC328STOP

5. DISCUSSION

In order to gain insight into the role of the C-terminal tail of the cGnRHR on its expression, the 55 amino acid C-terminal tail was truncated at S^{320}, R^{330}, S^{337}, S^{346}, T^{351}, D^{356}, S^{366}, and C^{375} [**figure 5.1**]. These truncated receptors were transiently transfected into COS-1 cells and tested for their maximal GnRH binding capacity, as an indication of their maximum receptor expression level. cS320STOP, the truncated cGnRHR that lacks the entire C-terminal tail did not display any binding or GnRH-induced IP production. This is consistent with results published for the C-terminal truncated rhodopsin receptor (Weiss *et al.*, 1994) and type 2 vasopressin receptor (Sadeghi *et al.*, 1997). No measurable binding could be detected for the naturally occurring C-terminal deficient mutant type 2 vasopressin receptor, V_2-R337STOP (Sadeghi *et al.*, 1997). Oksche *et al.* (1998) demonstrated by the use of fluorescence microscopy that the V_2-R337STOP mutant is produced in similar quantities as the full-length V_2-R. The V_2-R337STOP mutant however, does not reach the plasma membrane, but is trapped in the ER. The absence of the C-terminal tail from the V_2-R337STOP may have caused this receptor to be misfolded, resulting in the retention of this protein in the ER by a protein folding chaperone (s). Rozell *et al.* (1998) showed that the precursor forms of LHR and FSHR associate with the ER localized protein folding chaperone calnexin. These results suggest that some, if not all GPCRs interact with protein folding chaperones. Protein folding chaperones are believed to facilitate the proper folding of proteins and retain misfolded proteins in the ER (Rudden and Bedows, 1997). cR320STOP may also have been folded aberrantly and retained in the ER, resulting in the absence of GnRH receptor binding on the cell surface. Thus the C-terminal tail of the cGnRHR may play a role in stabilizing the proper receptor conformation and promoting receptor expression. Addition of the cfGnRHR intracellular C-terminal to the rGnRHR resulted in a 5-fold increase in receptor expression compared to the wt rGnRHR, which was reversible with truncation of the C-terminal tail from this chimeric GnRHR (Lin *et al.*, 1998). This suggests that the intracellular C-terminal tail of both of these non-mammalian GnRHRs [chicken and catfish] may possess certain elements that play an important role in receptor expression. It is reasonable to assume that these elements might be conserved between the catfish and chicken GnRHRs and possibly other non-mammalian GnRHRs.

cGnRHR : S^{320} FREDVQLCLR GIEAAISQHV RHKPISVSEK TTKDGDVNGQ VTSGGSNGTT VNTVC^{375}-COOH

cC328A : S^{320} FREDVQLALR GIEAAISQHV RHKPISVSEK TTKDGDVNGQ VTSGGSNGTT VNTVC^{375}-COOH

cC375STOP: S^{320} FREDVQLCLR GIEAAISQHV RHKPISVSEK TTKDGDVNGQ VTSGGSNGTT VNTV^{374}*

cS366STOP: S^{320} FREDVQLCLR GIEAAISQHV RHKPISVSEK TTKDGDVNGQ VTSGG^{365}*

cD356STOP: S^{320} FREDVQLCLR GIEAAISQHV RHKPISVSEK TTKDG^{355}*

cT351STOP: S^{320} FREDVQLCLR GIEAAISQHV RHKPISVSEK^{350}*

cS346STOP: S^{320} FREDVQLCLR GIEAAISQHV RHKPI^{345}*

cS337STOP: S^{320} FREDVQLCLR GIEAAI^{336}*

cR330STOP: S^{320} FREDVQLCL^{329}*

cS320STOP: *

Figure 5.1 List of primary sequences of the intracellular C-terminal tail of the full-length cGnRHR, point-mutated cC328A, and progressively truncated cGnRHR mutants. The termination of the protein sequence is indicated by *. The A replacing C^{328} in construct cC328A is depicted in green. Positively charged residues are depicted in blue and serine/threonine residues written in red.

In this study the cR330STOP mutant, which has a 9 amino acid C-terminal tail was the shortest truncated cGnRHR engineered that could be detected on the cell surface by binding studies. This suggests that the N-terminal region of the C-terminal tail, between P^{319} and G^{331} may be important in the proper folding and/ or expression of the cGnRHR. Interestingly, the N-terminal domain of the C-terminal tail in the cGnRHR has a high homology with that of other non-mammalian GnRHRs, including the catfish and goldfish type A and B [**figure 5.2**].

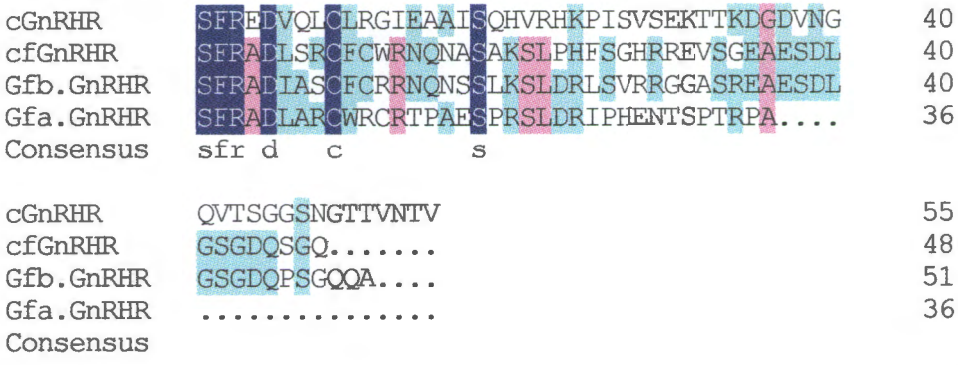


Figure 5.2 Alignment of primary sequences of the non-mammalian GnRHR C-terminal tails, including the chicken, catfish, and goldfish type A and B GnRHRs. All the residues in blue are identical, residues in pink have 75% conservation and the residues written in turquoise are 50% conserved.

The shortest length of intracellular C-terminal tail required for proper folding and expression is not known from our results, because no truncations were made between S^{320} and R^{330}. Nevertheless, these results suggest that the membrane proximal domain of the cGnRHR C-terminal tail may be required for proper receptor folding and expression. This is consistent with results published for the rat type 1a angiotensin receptor [AT_{1a}-R] (Gáborik *et al.*, 1998), bovine Rh-R (Weiss *et al.*, 1994) and type 2 vasopressin receptor (Sadeghi *et al.*, 1997; Oksche *et al.*, 1998). Gáborik *et al.* (1998) showed that replacement of F^{309} [located in the membrane proximal region of the AT_{1a}-R] with an alanine reduced the cell surface expression by 80%. This specific phenylalanine residue is highly conserved in most GPCRs, including the non-mammalian GnRHRs [chicken, catfish, goldfish type A and B GnRHR] as seen in **figure 5.3**. Therefore, it may be possible that F^{321} plays a similar role in expression of the cGnRHR, and that this function is conserved in all the non-mammalian GnRHRs.

AT_{1a}-R	K K F K K^{311}
RhR	K Q F R N^{315}
cGnRHR	P S F R E^{323}
cfGnRHR	P S F R A^{334}
GfaGnRHR	P S F R A^{323}
GfbGnRHR	P S F R A^{336}

Figure 5.3 Alignment of the membrane proximal domain of the C-terminal tail of the type 1A angiotensin receptor and bovine rhodopsin receptor with non-mammalian GnRHRs. A conserved phenyalanine [F] is depicted in red and the conservation of a positive charge in blue.

In addition, alanine replacement of the four lysine residues clustered around F^{309} [K^{307}, K^{308}, K^{310} and K^{311}] completely abolished expression of the AT_{1a}-R. A mutant containing alanines replacing both K^{310} and K^{311} had a 5 fold reduced receptor expression compared to the wt AT_{1a}-R (Gáborik *et al.*, 1998). Similarly, substitution of all the positively charged residues in the intracellular C-terminal tail of the V_2-R [R^{337}, R^{344}, R^{346} and K^{367}] with negatively charged glutamine residues, markedly reduced the cell surface receptor expression to 30% of the wt expression (Oksche *et al.*, 1998). These results suggest that positive charges contained in the intracellular C-terminal tails of GPCRs may be critical for receptor expression. Non-mammalian GnRHRs possess a highly conserved arginine residue [**figure 5.3**] at a position homologous to K^{310} of the AT_{1a}-R. This conserved arginine may therefore have a conserved role in promoting receptor expression of non-mammalian GnRHRs. The presence of F^{321} and R^{322} in the C-terminal tail of the cR330STOP-truncated mutant may therefore be responsible or contribute to the expression of this truncated receptor on the cell membrane. Our results show that the cR330STOP mutant has only 12% cell surface receptor expression relative to the wt receptor, indicating the involvement of additional elements in promoting receptor expression. Progressive increase in the length of the C-terminal tail of the cGnRHR progressively increased the cell surface receptor expression [**figure 4.1A** and **table 4.1**]. This suggests that these elements reside in the intracellular C-terminal tail downstream to L^{329} [**figure 5.1**]. The cGnRHR possesses five additional positively charged residues in its intracellular C-terminal tail, R^{330}, R^{341}, K^{343}, K^{350} and K^{353} as seen in **figure 5.1** that may also be

involved in promoting receptor expression on the cell surface (Gáborik *et al.*, 1998; Oksche *et al.*, 1998).

An increase in the length of the cGnRHR C-terminal tail from L^{329} to I^{336} [cS337STOP] resulted in more than two fold increase in receptor expression compared to cR330STOP [**figure 4.1A** and **table 4.1**]. R^{330} resides within this extended C-terminal tail region and is conserved between the chicken and type A goldfish GnRHR [**figure 5.2**]. This is the only positively charged residue present in the sequence that extends the C-terminal tail from cR330STOP to cS337STOP truncation and may therefore contribute to the increase in expression of cS337STOP. Another highly conserved residue that resides between L^{329} and I^{336} of the cGnRHR is C^{328} [**figure 5.2**]. This residue does not appear to be involved in promoting cGnRHR expression, because substitution of C^{328} with an alanine did not impair receptor expression, but yielded a two fold higher expression than the wt cGnRHR [**figure 4.5A** and **table 4.4**]. C^{328} can therefore not be responsible for the ~18% higher receptor expression of cS337STOP compared to cR330STOP. Further extension of the C-terminal tail from I^{336} to I^{345} [cS346STOP] resulted in two fold higher expression of cS346STOP compared to cS337STOP [**figure 4.1A** and **table 4.1**]. Two positively charged residues [R^{341} and K^{343}] are present in this 9 amino acid extension, of which the positive charge of K^{343} is conserved amongst the non-mammalian GnRHRs as seen in **figure 5.2**.

No further increase in receptor expression was observed with extension of the cGnRHR C-terminal tail from I^{345} to K^{350} [cT351STOP] or K^{350} to G^{355} [cD356STOP]. It is noteworthy though that each of these two receptor extensions possesses a positively charged residue, K^{350} and K^{353} respectively of which neither is conserved. A further increase in expression was obtained with extension of the C-terminal tail from G^{355} to G^{365}, and wt levels of expression reached with increase in length of the C-terminal tail from G^{365} to V^{374} [**figure 4.1A** and **table 4.1**]. The sequence between G^{355} and C^{375} does not contain any positively charged residues, yet the presence of this sequence improved receptor expression to wt levels [**figure 4.1A** and **table 4.1**]. These results indicate that other sequences or elements present in the C-terminal tail beyond G^{355}, in addition to positively charged residues might play a role in promoting receptor expression of the cGnRHR. Truncation of C^{375}, the last amino acid from the cGnRHR C-terminal tail, did not alter the cell surface expression, indicating that C^{375} does not play a role in receptor expression. All the truncated

cGnRHRs, as well as the cC328A mutant had affinities in the range of 2nM to 7nM, which is similar to that of the wt. This indicated that truncation of the cGnRHR C-terminal tail from R^{330} and point mutation of C^{328} to alanine did not alter the tertiary structure of the cGnRHR. No universal consensus sequence for promoting GPCR expression is known. Therefore it is difficult to suggest which residues in the distal part of the cGnRHR C-terminal tail are responsible for increasing receptor expression to wt levels. The contrasting finding that mammalian GnRHRs, which don't have a C-terminal tail, are expressed while the cGnRHR is not expressed without a C-terminal tail, suggests that mammalian GnRHR expression is dependent on different domains within the receptor.

Newly synthesized proteins are mainly sorted and packaged for their different destinations in the trans-Golgi network [TGN]. It has been shown that proteins are tagged with information in their primary sequence that dictates their final destinations. Proteins destined to reside in the ER are tagged with a KDEL motif (Pelham, 1990; Vaux *et al.*, 1990), whereas proteins destined for delivery to lysosomes are tagged with a mannose-6-phosphate unit (von Figura and Hasilik, 1986). The use of a monoclonal antibody against the Rh-R C-terminal tail facilitated in implicating the C-terminal tail in sorting and trafficking of newly synthesized Rh-R to the rod outer segment membrane (Deretic *et al.*, 1996). This antibody recognizes the last 9 amino acids on the C-terminal of the Rh-R. Formation of post-Golgi vesicles containing newly synthesized Rh-R were severely inhibited by this antibody in a cell-free [membrane extract] assay. Deretic *et al.* (1998) demonstrated that peptides corresponding to the last 24 amino acids of the frog and bovine Rh-R C-terminal tail have a similar inhibitory effect. These peptides significantly impaired post-Golgi trafficking and trapped newly synthesized Rh-Rs in the TNG. Post-Golgi trafficking was repaired with truncation of the last 5 amino acids [QVS(A)PA] from these peptides. These residues are highly conserved at the end of the C-terminal tail of the Rh-R family and may therefore be required for post-Golgi vesicle formation and Rh-R trafficking. This sequence motif is suggested to serve as a sorting sequence recognized in the trans-Golgi by factors involved in transport of the Rh-R out of the trans-Golgi to post-Golgi vesicles (Deretic D., 1998; Deretic *et al.*, 1998). This sorting signal is not conserved amongst GPCRs outside the Rh-R family. Nevertheless, it may be possible that the C-terminal tail of most GPCRs are tagged with a sorting signal, considering the evidence implicating the C-terminal tail in

GPCR expression. Though very little is known about the trafficking of newly synthesized GPCRs, it is apparent that more than one sequence motif in the intracellular C-terminal tail governs GPCR expression. This may very well be true for the cGnRHR and other non-mammalian GnRHRs (Lin *et al*, 1998).

All the truncated cGnRHRs were tested for their ability to generate second messenger IP_3 in response to CI GnRH. This was done in order to determine whether these truncated receptors are functional. All the truncated mutants were active, except cS320STOP, which was therefore not further investigated [**figure 4.2A**]. Progressive truncation of the cGnRHR C-terminal tail from the last residue, C^{375} to S^{346} resulted in truncated receptors that generate greater levels of IPs than the full-length cGnRHR. Interestingly, all these truncated receptors had a lower cell surface expression than the wt cGnRHR. The highest maximal GnRH-induced IP production was generated by cD356STOP. This truncated cGnRHR produced an almost two-fold greater IP yield [~191%] than the wt, yet its cell surface expression is approximately half [~45%] that of the wt. It therefore appears as though truncation of the cGnRHR C-terminal tail between I^{345} and C^{375} improved coupling efficiency. Ballesteros *et al.* (1998) derived a formula to determine coupling efficiency [described in **methods 3.5**] and used it to compare coupling efficiency between point mutated hGnRHRs and their wt counterpart. By applying this formula, we indeed established that all the truncated receptors between I^{345} and C^{375} have greater coupling efficiency values than the full-length cGnRHR. In addition, we found that cS337STOP and cR330STOP also have coupling efficiency values greater than the wt cGnRHR. These results suggest that the cGnRHR couples more efficiently to its cognate G-protein in the absence of its intracellular C-terminal tail. Similar results were obtained for the type 2 histamine receptor [H_2-R] where partial truncation of its C-terminal tail enhanced second messenger cAMP production (Fukushima *et al.*, 1997). Interestingly, it was found that progressive shortening of the β-type prostaglandin E receptor [EP3β-R] C-terminal tail resulted in partial to complete constitutive activation of the resultant mutant receptors (Hizaki *et al.*, 1997). Hizaki *et al.* (1997) proposed that the C-terminal tail of the EP3β-R plays a role in constraining this receptor into its inactive conformation. The level of constitutive activation was inversely related to the length of the EP3β-R C-terminal tail. No constitutive activation was detected for any of our truncated

cGnRHRs. Nevertheless, it might be possible that the C-terminal tail of the cGnRHR also plays an inhibitory role in receptor/G-protein coupling by masking intracellular domains that couple to $G\alpha_{q/11}$. Truncation of the C-terminal tail of the cGnRHR would thus more readily expose such domains to $G\alpha_{q/11}$ and improve coupling efficiency. This would imply that the C-terminal tail of the cGnRHR distal to R^{330} might not be directly involved in promoting receptor/G-protein coupling. No truncations were made between S^{320} and R^{330} of the cGnRHR C-terminal tail. However, replacement of the highly conserved C^{328} with alanine impaired receptor/G-protein coupling, suggesting that this C^{328} might be involved in coupling of the cGnRHR to $G\alpha_{q/11}$. The membrane proximal domain of the C-terminal tail generally contains determinants that contribute to G-protein coupling (O'Dowd *et al.*, 1988; Cotecchia *et al.*, 1990). Thus, the N-terminal domain of the cGnRHR C-terminal tail, between S^{320} and R^{330} can therefore not be ruled out as a possible G-protein-coupling domain.

Intuitively, one would expect that a mutant receptor [for example cD356STOP] that has a significantly lower expression, but greater coupling efficiency than its wt, would have a left-shifted EC_{50} [improved potency]. Instead, none of our truncated cGnRHRs had improved potencies. All the truncated cGnRHRs have EC_{50} values similar to that of the full-length cGnRHR, indicating that truncation of the C-terminal tail beyond L^{329} did not alter the receptor potency [**figure 4.2B** and **table 4.2**]. This suggests that the enhanced levels of IPs generated by the truncated receptors might not be due to improved coupling efficiency *per se*, if at all. It might be possible that the coupling efficiencies of the truncated cGnRHRs did not improve at all, but that alternate factors are responsible for their robust IP production. Such factors would include the difference in the rate of internalization and desensitization between the truncated receptors and the wt. This is not taken into consideration in the coupling efficiency equation derived by Ballesteros *et al.* (1998).

Pawson *et al* (1998) demonstrated that the cGnRHR undergoes rapid internalization at a rate of 11.3% per minute. Truncation of the C-terminal tail at S^{337} [cS337STOP] severely reduced the GnRH-induced receptor internalization to 0.55% per minute. These results implicated the C-terminal tail of the cGnRHR in mediating rapid internalization. Similarly, the C-terminal tail of the H_2-R has been shown to be important for mediating rapid internalization (Fukushima *et al.*, 1997). Truncation of

the last 51 amino acids from the H_2-R C-terminal tail abolished rapid internalization without affecting desensitization. In addition, the resultant mutant, T^{308} yielded a cAMP production that was 1.5 times higher than that of the full-length H_2-R. The absence of internalization of the T^{308} mutant allowed this receptor to be retained on the cell surface much longer than its wt counterpart. This might permit a longer time-period for receptor/G-protein interaction. The cAMP production of the T^{308} mutant would therefore be more robust than the wt H_2-R. This might also explain the enhanced IP production of the truncated cGnRHRs. The truncated cGnRHRs might have a higher retention time on the cell membrane, allowing a longer period of receptor /G-protein coupling. Thus resulting in an enhanced level of IP production from the truncated cGnRHRs compared to the full-length cGnRHR.

The specific determinants in the cGnRHR C-terminal tail responsible for mediating rapid internalization are yet to be identified. Nevertheless, the C-terminal tail of the cGnRHR is rich in serine/threonine residues, possessing 6 serines and 6 threonines [**figure 5.1**]. Serine/threonine residues have been shown to play a role in promoting rapid internalization of various GPCRs (Benya *et al.*, 1993; Fukushima *et al.*, 1997; Böhm, 1997). After agonist stimulation, serine/threonine residues in the C-terminal tail of the activated GPCR are phosphorylated by G-protein-coupled receptor kinases [GRKs]. GRKs are cytosolic protein kinases that are recruited to activated GPCRs in the plasma membrane. Arrestin molecules bind to the phosphorylated serine/threonine regions and are believed to serve as adapter molecules that allow interaction between the internalization machinery and the GPCR (Ferguson *et al.*, 1996). Progressive shortening of the cGnRHR resulted in the loss of serine/threonine residues with each truncation, except for cC375STOP. The cR330STOP and cS337STOP did not display enhanced levels of IP production [**figure 4.2A** and **table 4.2**], but greater coupling efficiency values than the full-length cGnRHR [**figure 4.3** and **table 4.3**]. However, expression of cR330STOP and cS337STOP was reduced eight and three fold respectively [**figure 4.1A** and **table 4.1**]. Their maximal IP production might therefore be more robust than that of the wt cGnRHR, if their cell surface expression was equivalent to the wt.

Phosphorylation of serine/threonine residues in the C-terminal tail of many GPCRs by GRKs is also associated with rapid desensitization. Arrestin molecules bind to these phosphorylated areas, prevent receptor/G-protein coupling and desensitizes the

activated receptor (Böhm, 1997). The cfGnRHR has been demonstrated to internalize and desensitize rapidly in HEK-293 cells, which is most probably mediated via its intracellular C-terminal tail that possesses nine serines (Heding *et al.*, 1998). Addition of the cfGnRHR C-terminal tail to the rGnRHR induced the chimeric receptor to undergo phosphorylation and desensitize rapidly in response to GnRH (Willars *et al.*, 1999). It is not yet known whether the cGnRHR undergoes rapid desensitization. Considering that the cfGnRHR desensitizes rapidly and that the cGnRHR C-terminal tail is also rich in serine/threonine residues, it might be possible for the cGnRHR to desensitize rapidly. Rapid internalization and desensitization has been shown to occur independently via different molecular determinants in the C-terminal tail of GPCRs, including AT_{1a}-R (Thomas *et al.*, 1995) and H_2-R (Fukushima *et al.*, 1997). Of the thirteen serine/threonine residues present in the H_2-R, only T^{315} was found to be involved in rapid internalization, but not rapid desensitization of the H_2-R. It might therefore be possible that several of the serine/threonine residues present in the cGnRHR C-terminal tail are involved in rapid internalization of this receptor, while other serine/threonine residues present in the C-terminal tail might be required for rapid desensitization. Progressive truncation of the cGnRHR C-terminal tail might have reduced or abolished rapid desensitization. This would prevent or slow down the attenuation of signaling, explaining the enhanced maximal IP production by the truncated cGnRHRs. Truncation of the NK_2-R abolished rapid desensitization and resulted in an enhanced IP production (Alblas *et al.*, 1995). Thus, the absence of rapid internalization and desensitization might cooperatively or independently have been responsible for the robust IP production from the truncated cGnRHRs.

Most GPCRs have one or more cysteine residues in the membrane proximal domain of their C-terminal tail that is palmitoylated, including the V_2-R (Schülein et al., 1996) and α_{2A}-R (Kennedy and Limbird, 1993). This post-translational modification has been implicated in regulatory processes, including G-protein coupling, internalization and desensitization of various GPCRs (Böhm *et al*, 1997). C^{328} of the cGnRHR is highly conserved amongst the non-mammalian GnRHRs, including the type A and B goldfish GnRHR and the cfGnRHR [**figure 5.2**]. Our results show that mutation of C^{328} to alanine [cC328A] increased the expression two fold, while reducing the

maximal IP production and coupling efficiency to ~69% [**figure 4.5C** and **table 4.4**] and 0.3 [**figure 4.5E** and **table 4.4**] respectively.

This reduced IP production and coupling efficiency value suggest that the cGnRHR-$G\alpha_{q/11}$ coupling was severely impaired. Horstmeyer *et al.* (1996) replaced a cluster of five cysteine residues in the C-terminal tail of the type A endothelin receptor [ET_A-R], proximal to the membrane with alanines and serines respectively. This abolished palmitoylation and IP production without affecting the cAMP production. These cysteines are conserved in the type B endothelin receptor [ET_B-R] and substitution of three of them by serines abolished palmitoylation and coupling of this receptor with both $G\alpha_i$ and $G\alpha_q$ (Okamoto *et al.*, 1997). These results suggest that palmitoylation in some GPCRs might play a role in G-protein coupling. The severe reduction in IP production from cC328A indicates that this residue might be involved in receptor/G-protein coupling. Furthermore, substitution of C^{341} with glycine in the β_2-AR C-terminal tail abolished palmitoylation, severely impaired cAMP production and increased basal levels of receptor phosphorylation in the C-terminal tail (Moffett *et al.*, 1996). Substitution of S^{345} and S^{346} by alanines in the C341G β_2-AR mutant reduced basal phosphorylation and restored receptor-$G\alpha_s$ coupling to that of the wt β_2-AR. The increased basal levels of phosphorylation uncoupled the C341G mutant from $G\alpha_s$ resulting in reduced cAMP production. Moffett *et al.* (1996) suggested that palmitoylation of the wt β_2-AR constrains its C-terminal tail into a conformation that reduces the accessibility of S^{345} and S^{346} to protein kinase A [PKA]. This prevents phosphorylation at these two serines, allowing the receptor to couple normally to $G\alpha_q$. This might also be applicable to the cGnRHR, provided that this receptor undergoes desensitization. The replacement of C^{328} with alanine may also increase basal phosphorylation levels of this mutant cGnRHR, promoting the onset of receptor phosphorylation and uncoupling prior to receptor activation. No direct evidence exists to prove that C^{328} of the cGnRHR is palmitoylated. However, the conservation of this residue amongst non-mammalian GnRHRs and the impairment in receptor/G-protein coupling from cC328A, suggests that the cGnRHR might be palmitoylated at C^{328}. Palmitoylation of this residue might serve a structural purpose that facilitates coupling or plays a role in receptor phosphorylation.

In conclusion, complete truncation of the cGnRHR C-terminal tail abolished agonist binding and GnRH-induced IP response, whereas progressive truncation reduced receptor expression. This suggests that the C-terminal tail of the cGnRHR might be required for cell surface expression of the cGnRHR by playing a role in trafficking. Mutation of C^{328} to alanine and shortening of the cGnRHR C-terminal tail might not have altered the tertiary structure of the resultant mutant receptors since receptor affinity did not change. In addition, the membrane proximal region of the C-terminal tail may play a role in the proper folding of the newly synthesized receptor. The enhanced IP and coupling efficiency values indicate that shortening of the C-terminal tail might improve coupling. However the similarity in EC_{50} values between the truncated and full-length cGnRHR suggest that alternate processes might be responsible for the enhanced IP response. These include a possible loss or reduction in the rate of internalization and/or desensitization with truncation of the C-terminal tail. Substitution of the highly conserved C^{328} with alanine severely impaired coupling, suggesting that the cGnRHR C-terminal tail might be palmitoylated at C^{328} residue.

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